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STAPHYLOCOCCI AND SALMONELLA CONTROL IN FOODS[1]

I. EFFECT OF ULTRA-VIOLET RADIATION AND OZONE ON BAKERY CUSTARD AND CREAM FILLINGS

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The laboratories of the American Institute of Baking are working on methods of controlling certain bacteria in food products—specifically in bakery products containing custard and cream fillings. The work has been confined to staphylococci and Salmonella bacteria since these are the two types of bacteria chiefly reported as causative agents in food-illness cases.

Stritar, Dack, and Jungewaelter (1936a) have reported the early work on a reheating (pasteurization) method for controlling staphylococci in custard-filled puffs and éclairs. They also found that Salmonella could be controlled by the same methods as outlined for staphylococci (1936b).

Although this reheating method offers a means of sterilizing puffs and éclairs after they have been filled, it is not applicable to all types of cream-filled products and it is possible for the products to become contaminated after reheating. The refrigeration of puffs and éclairs from time of reheating until consumption should offer a complete control, provided the baker exercises proper care before reheating. However, the education of *all* consumers in the necessity of proper refrigeration in the home is practically impossible.

Owing to the limitations of the above method, other methods of control are being studied. The work reported in this paper was undertaken to determine if ultra-violet radiation or ozone could prevent contamination of the particular type of products concerned when the source of contamination was air-borne bacteria.

Ultra-violet radiation with wave lengths of the order of 2,000 to 2,950 Angström units has been found successful for killing molds and bacteria under prescribed conditions and is being used for maintaining sterility in the storage of certain food products. Broadbent (1938) reports that radiation of this nature will kill *Staphylococcus aureus* in 23 to 28 seconds when the sample is four inches from the lamp. Owing to the fact that radiations of these wave lengths will not penetrate even thin films of most substances they are of no value in the sterilization of food products. It is generally known that their value lies in the fact that they will kill organisms in the air and on smooth, exposed surfaces, provided the organisms themselves are exposed to the rays for a sufficient length of time.

[1] Funds for research reported in these papers were granted by the Joint Committee on Food Protection of the American Bakers Association and the Associated Bakers of America.

Ozone has been used in some industries as a bactericidal agent. The literature in regard to the use of ozone in food storage has been discussed by Ewell (1938).

EXPERIMENTAL PROCEDURE

An atmosphere of the particular bacteria being studied was created by spraying an inoculum of the organism into a specially constructed box by means of a nasal atomizer through small holes in the ends of the box near the top. Any fog created in the box by the atomizer became invisible instantaneously.

The box was 42 inches long, 24 inches high, and 20 inches wide and was made of ply wood. There was an opening three inches wide, the length of the side (near bottom), fitted with a door for inserting samples. Another opening along the center of the top was designed to accommodate the lamps. When ozone was being studied, the box was completely closed except for the small holes through which the organisms were sprayed. A commercial-type ozone generator, with circulating fan, was fastened to the top, inside the box, when used. The purpose of the box was to simulate show-case conditions.

Both blood agar and standard nutrient agar plates were used for determining the direct efficiency of the lamps and the ozone. These plates were placed in the bottom of the box before the atmosphere of organisms was created, and the plates were exposed to this atmosphere for varying lengths of time.

The "pies" were made by pouring the custard (cooked two to four minutes after second boil) into sterile Petri plates. The plates were then placed open in an oven at 274°C.(525°F.) until a "skin" had formed on the surface of the custard. They were then cooled under as sterile conditions as possible and immediately used. This procedure gave a surface exactly comparable to the surface of commercially prepared custard pies.

Unless otherwise stated "spread" plates were made by spreading as smoothly as possible a prepared custard (three ounces of starch) into sterile Petri plates.

The custard for the puffs and "spread" plates were prepared from the following formula:

Water	1 qt.
Dried whole milk	4 oz.
Sugar (sucrose)	8 oz.
Frozen whole eggs	4 oz.
Cornstarch	3 oz.
Salt	⅛ oz.

The puffs were made in the usual manner, filled from the top[2] and subjected to the pasteurization treatment as described by Stritar, Dack, and Jungewaelter (1936a) before being used.

Both a hemolytic strain of *Staphylococcus aureus* and a strain of *Salmonella enteritidis* were used. Salmonella organisms were used in spite of the fact that there is no evidence of an infection ever having been

[2] Puffs which are to be subjected to the reheating (pasteurizing) treatment must be filled from the top or near the top.

brought about by air-borne Salmonella. Both of these cultures were isolated from food-illness cases.

Four to five Petri plates, puffs, "pies" or "spread" plates were used in each test, incubated for 24 hours at 36.7 to 37.2°C.(98 to 99°F.) (in some experiments the "spread" plates were plated immediately after exposure), the contents then mixed, and a 25-gram sample taken from the composite (only custard of puffs was used). This sample was then mixed with 100 milliliters of sterile water by means of an electric hand mixer and dilutions were made from this emulsion. Decimal dilutions of this emulsion were plated using blood agar plates for staphylococci and nutrient agar plates for Salmonella.

RESULTS AND DISCUSSION

Direct Killing Power of Ultra-Violet Radiation: Both blood agar and standard nutrient agar plates were exposed to an artificial atmosphere of organisms (estimated to be about 1,000,000 organisms) in the box with and without the ultra-violet rays. By taking the plates out at varying lengths of time the efficiency of the radiation from various lamps in killing the organisms in the atmosphere was determined; a comparison of the efficiency of the lamps used is shown (Table 1). All three were mercury vapor lamps. Lamp No. 1 was a unit containing two 34-inch tubes, lamp No. 3 contained only one 34-inch tube, and lamp No. 2 contained one tube five inches long. Lamp No. 2 generated considerable heat, while the other two lamps operated relatively cool.

TABLE 1

Comparison of Efficiency of Three Standard Ultra-Violet Ray Lamps

Lamp	Staphylococci	Salmonella
1	2½ min.—complete kill	8 min.—nearly complete kill
2	20 min.—a few organisms remaining	45 min.—a few organisms remaining
3	4 min.—complete kill	8 min.—nearly complete kill

Plates exposed to an atmosphere of staphylococci under lamp No. 1 for zero, one, and two and one-half minutes, respectively, after 24 hours' incubation, are shown (Fig. 1A); the same for Salmonella, except for periods of zero, two, and eight minutes, respectively, are shown (Fig. 1B). Observations indicate that (1) it takes a relatively short time to kill either of these two organisms with an efficient lamp; and (2) it takes longer to kill Salmonella than staphylococci.

Direct Killing Power of Ozone: Both blood agar and standard nutrient agar plates were exposed to an atmosphere of organisms (estimated to be about 1,000,000 organisms) in the box, for varying periods of time with ozone and without ozone. The exposed plates were then incubated for 24 hours at 36.1 to 36.7°C.(97 to 98°F.). A concentration of four parts per million of ozone was built up prior to creating the atmosphere and inserting the plates. Higher concentrations of ozone than this were not used because it was felt that they would be impracticable. For instance, if this were to be used in an ordinary show case, a greater con-

centration than four parts per million would allow enough ozone to escape to be noticeable to the customers.

Plates which were exposed, after incubation, to an atmosphere of staphylococci with four parts per million of ozone present for ½, 27, and 60 minutes, respectively, are shown (Fig. 2A); the same for Salmonella, except for periods of 2, 25, and 26 minutes, respectively, are shown (Fig. 2B). It will be noted that after 60 minutes some reduction in the number of organisms has taken place, yet the reduction is not sufficient.

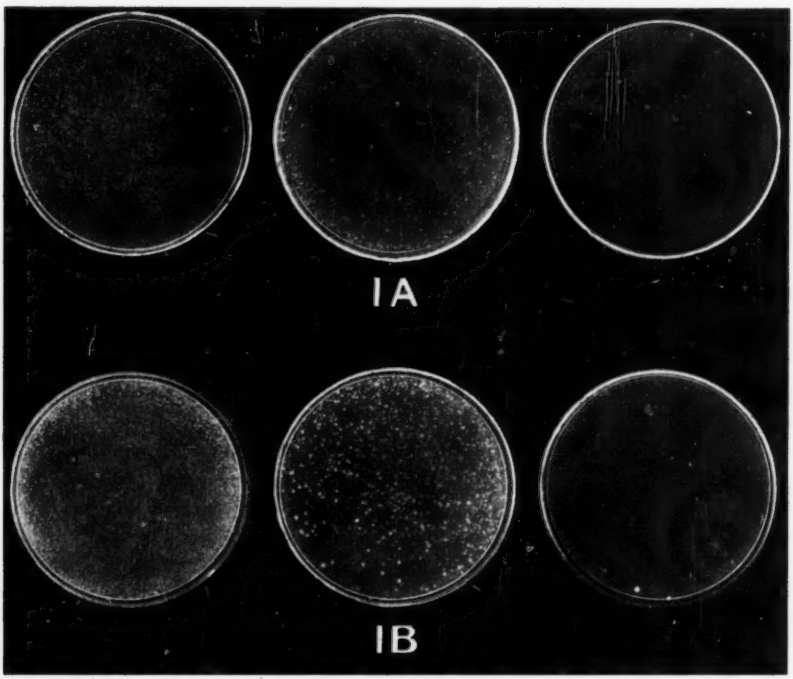

FIG. 1. 1A shows plates which have been exposed to an atmosphere of staphylococci under lamp No. 1 for zero, one, and two and one-half minutes (reading from left to right). 1B shows same for Salmonella, except that the time periods were zero, two, and eight minutes, respectively.

Even in experiments where the total number of organisms inoculated into the box was as low as 27 (accurate count on inoculum) there was little reduction in organisms after 60 minutes. Thus, in the concentrations used (and these concentrations tended to become obnoxious) ozone would not be effective in helping reduce the chance of contamination of food (especially bakery custard creams) from staphylococci and Salmonella bacteria. Therefore, custard cream products were not used in experiments with ozone.

Effect of Ultra-Violet Radiation on Custard Products: "Spread" plates were exposed to each of the three lamps for 20 minutes in an

atmosphere of staphylococci, but only the most efficient lamp (No. 1) was used (for 20 minutes) with the "pies" and puffs. "Spread" plates and "pies" were exposed to lamp No. 1 in an atmosphere of Salmonella. The bacterial counts of the above as compared with counts on the same products not exposed to the rays are shown (Table 2).

Accurate counts were not made of the number of organisms in the box atmosphere, but it is estimated that about 1,000,000 organisms were sprayed into the box. As a result comparisons must be made between the

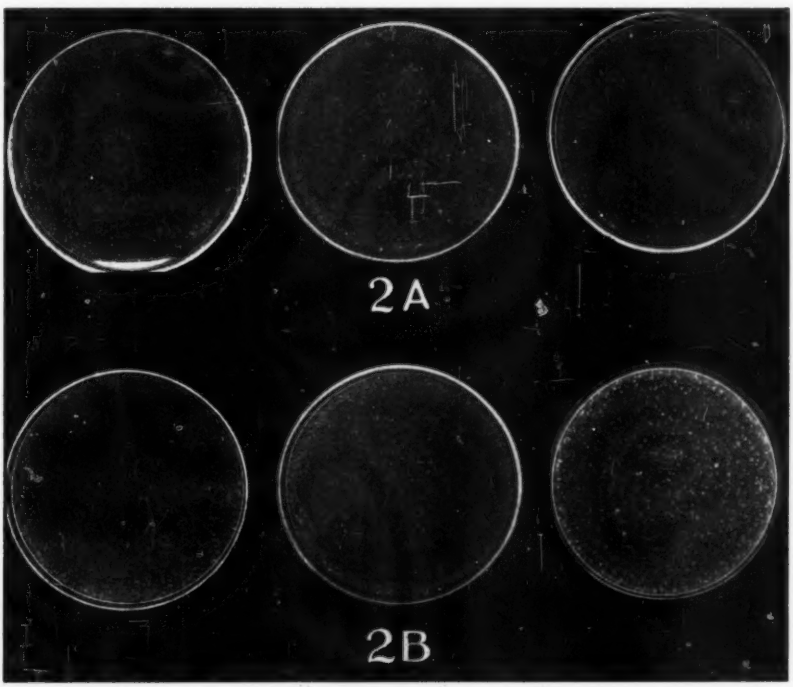

FIG. 2. 2A shows plates which have been exposed to an atmosphere of staphylococci in an atmosphere of four p.p.m. ozone for ½, 27, and 60 minutes (reading from left to right). 2B shows the same for Salmonella, except that the time periods were 2, 25, and 60 minutes, respectively.

counts in each series rather than making a comparison of all the counts. Comparisons of reductions in various series can, of course, be made. Agar plates were used in each series, being placed alongside each set of samples, and these gave an indication of the number of organisms in the atmosphere as well as a recording of the efficiency of the lamp at that particular time. Comparisons of pictures of these plates show that the atmosphere varied somewhat, but the efficiency of the lamps remained about the same over the period of the experiments.

It can be seen from the data on "spread" plates (Table 2) that efficiencies of the lamps are of the same order as previously indicated by

the directly exposed agar plates (Table 1). The "spread" plates having no "skin" and a very rough surface were much more susceptible to inoculation than the "pies" (Series 4, Table 2), since the bacteria could easily fall into a crevice where the ultra-violet rays could not penetrate. This, therefore, accounts for the higher counts on "spread" plates over "pies" exposed to the radiation. This same reasoning will account for the greater effectiveness of the lamps, when their direct efficiency was determined by means of agar plates.

The data indicate first, that ultra-violet radiation is more effective on staphylococci than on Salmonella, and second, that it is more effective on "pies" than on "spread" plates.

TABLE 2

Counts on Various Custard Products Exposed to an Atmosphere of Organisms for 20 Minutes With and Without Ultra-Violet Radiation[1]

Series	Type of product	Organisms per gram of custard			
		Staphylococcus aureus		*Salmonella enteritidis*	
		Exposed to rays	Not exposed to rays	Exposed to rays	Not exposed to rays
1	"Spread" plates—lamp No. 1	11,350,000	326,000,000	.....	.....
1	"Spread" plates—lamp No. 2	219,500,000	326,000,000	.....	.....
1	"Spread" plates—lamp No. 3	28,350,000	326,000,000	.....	.....
4	"Spread" plates—lamp No. 1	.....	.....	46,250,000	50,500,000
2	"Pies"—lamp No. 1	4,850,000	388,500,000	.....	.....
4	"Pies"—lamp No. 1	.....	.....	1,593,000	11,775,000
3	Puffs, incubated in covered jars—lamp No. 1	702,500	2,755,000	.....	.....
3	Puffs, incubated uncovered—lamp No. 1	56,500	178,500	.....	.....
5	Puffs, incubated uncovered—lamp No. 1	34,000	107,000	.....	.....

[1] Products were incubated for 24 hours at 36.7 to 37.2°C. (98 to 99°F.) after exposure.

The "pasteurized" puffs were first incubated in jars in an attempt to maintain sterility, but the shells became so soggy under these conditions that it was decided to incubate them openly (uncovered) as they would be exposed in a show case. When this was done, the shells remained in a crisp, dry condition. Apparently owing to the ease with which the organisms on the surface penetrated the moist shell, the counts for both the exposed and the unexposed puffs were much higher in the cases where the puffs were covered during incubation. The lower reduction in the case of puffs as compared with the "pies," using staphylococci, was undoubtedly due to the fact that the puff shells have many crevices into which the organisms may fall and into which the rays of the lamp cannot penetrate.

In order to be sure in the previous experiments that inoculums were not so high that clouds of organisms masked the ultra-violet radiation, further experiments were run with sterilized "spread" plates using *Staphylococcus aureus*. Counts were made on the inoculum and the number of organisms sprayed into the box was calculated; these data are given (Table 3).

With these low inoculations, organisms are found in the custard; however, the lamp effected large reductions. Also, most "spread" plates not exposed to ultra-violet rays showed more organisms per gram, when plated immediately, than the total number inoculated into the box. This same observation was noted in experiments with ozone; however, no explanation is offered.

Development of Off-Aroma and Off-Taste: It was also observed during the course of these experiments that an off-aroma and taste develops in the products exposed to the rays of the lamps used in the tests. The custard developed a rancid, fishy aroma when exposed to the direct radiation of these lamps for two hours or longer. Bread, butter, cakes, puff shells, and cake batters were also found to develop this off-aroma. It seemed gradually to leave the product after removal from the rays. It is reported

TABLE 3

Counts on Sterilized "Spread" Plates Exposed to an Atmosphere of Staphylococcus aureus for 20 Minutes With and Without Ultra-Violet Radiation [1]

Number of inoculum organisms sprayed in box	Number of organisms per gram			
	Exposed to rays		Not exposed to rays	
	Plated immediately	Plated after 24-hour incubation	Plated immediately	Plated after 24-hour incubation
27	0	....	105	.....
6,030	15	....	75	21,200,000
12,600	0	0	218,000	650,000,000
201,000	15	65	5,400,000	255,000,000

[1] Lamp No. 1 was used.

that this same difficulty has been experienced in the use of ultra-violet radiation in meat storage.

The manufacturers of these lamps have recognized this problem and are working on means of correcting it. It seems to be agreed among the lamp manufacturers that this off-aroma and taste are due to the ozone produced by wave lengths in the neighborhood of 1,800 Å units and/or, directly by radiations around 3,100 Å units. It is claimed that lamps in which the radiation is confined to the neighborhood of 2,500 to 3,000 Å units will produce no marked off-aroma or off-taste.

The authors made tests in an attempt to determine if this off-aroma in custard products is due to the ozone produced. Two lamps were used, one of which produced very little ozone, the other produced ozone. Products exposed to the first lamp required a longer time to develop the aroma but did develop it. This seems to indicate that a substantial amount of the off-aroma is due to ozone, but that some other factor, as mentioned above, may produce this effect directly.

No difficulty should be experienced in the use of these lamps in maintaining sterile conditions during the preparation of the custard products; however, in show cases where the products may lie for a longer period of time, this factor must be taken into consideration. It may be possible to

avoid this by sterilizing the air with these lamps as it enters the show case and thus avoid direct radiation of the products.

Samples of bread (both cut and uncut), sweet rolls, butter, and custards were placed in the box with an atmosphere of ozone (four parts per million) created by the ozonizer. The samples were removed from the box and tested for aroma and taste at various time intervals.

An off-aroma and taste of the same type noticed with the ultra-violet radiations was definitely detectable after one hour. The ozone used in these experiments seemed to have a greater effect upon taste than the ultra-violet radiation. Although the aroma tended to leave the product when standing in normal atmosphere, the taste persisted for some time. In the custard which was exposed to an atmosphere of ozone for one hour and then let stand for 40 hours in a normal atmosphere, both the off-aroma and off-taste had nearly disappeared. These experiments were repeated many times. These data seem to add to the more or less established fact that ozone is one of the main contributing causes of off-aroma from ultra-violet radiation intended to destroy bacteria.

SUMMARY

Ultra-violet rays (2,000 to 2.950 Å units) effectively reduced the counts of *Staphylococcus aureus* and *Salmonella enteritidis* in the air and on smooth surfaces; however, they would not penetrate the surface of custard products.

Baked custard "pies" and custard-filled puffs were exposed to an atmosphere of these organisms, with and without ultra-violet radiation. The radiation was found to reduce the degree of contamination of these products from the atmosphere.

Ultra-violet radiation was found to be more effective on smooth-surfaced "pies" than on irregular-surfaced puffs.

Lamps producing these rays will aid in maintaining a sterile atmosphere but are of no value in sterilizing these products. However, they are only an aid since an inoculation of bacteria into the air may contaminate the products before the bacteria are killed.

An off-aroma was found to be present in custard products exposed to these lamps for two hours or more.

Ozone was ineffective in helping to control staphylococci and Salmonella bacteria in custard and cream fillings. It was found to be ineffective first, because of its low rate of killing in the concentration in which it could be used; and second, because of the development of an off-aroma in such products as bread, rolls, custard, and butter.

The type of off-aroma and taste developed in the products by the ozone is the same as that developed by ultra-violet radiation of the type used for killing bacteria.

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STAPHYLOCOCCI AND SALMONELLA CONTROL IN FOODS
II. EFFECT OF PURE FRUIT FILLINGS

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Since it has been brought to the attention of the authors that many bakers believe pure fruit filled foods are not involved in outbreaks of food illness, and since only one record of a food-illness outbreak which could be traced to pure fruit fillings [1] could be found by the authors, an attempt to prove or disprove this contention was made.

The type of pure fruit filling considered is that where the fruit is ground or crushed and thoroughly mixed with the rest of the filling. A filling where the fruit is left whole, as in some fruit cream pies, is not to be considered a pure fruit filling as referred to in this paper. The reason these whole fruit fillings were not considered is that the fruit acids do not have an opportunity to mix with the filling and exert their effect on the bacteria.

Stritar, Dack, and Jungewaelter (1936) have found that citric acid had a decided inhibiting effect on a strain of *Staphylococcus aureus*, isolated from a food-illness case, when the organisms were added to custard. Seven and one-half grams of citric acid per quart of mix (approximately .54 per cent) prevented growth at the end of 48 hours, while concentrations above this had a decided killing action. However, these concentrations were detrimental to the taste of an ordinary custard. pH values were not given.

Hucker and Haynes (1937) studied the effect of acetic acid on strains of *Staphylococcus aureus*, of the type associated with food-illness cases, in veal infusion broth. They found that .15 per cent at the end of 48 hours brought about a decided reduction in the number of organisms.

Prescott and Tanner (1938) state that fruits and vegetables having pronounced natural acidity tend to delay, or in some cases inhibit, toxin production at 20°C.(68°F.) by *Clostridium botulinum*. These authors also refer to unpublished work of Wallace and Tanner in which it was found that death rates of microörganisms increased with increasing acidity (pH 9.6-4.0).

Since acidity has an inhibiting effect, it was thought that acid fruits in fillings should also show this effect to an extent depending on the amount and kind of fruit acids present. Thus, the effect of lemon, pineapple, orange, strawberry, and apricot fillings on inhibiting the growth of staphylococci and Salmonella bacteria was studied. Also standard custards with varying amounts of added lemon juice and rind were tested.

[1] One case of pineapple jelly (England) is reported by Fuller (1938).

EXPERIMENTAL PROCEDURE

Lemon, orange, pineapple, strawberry, and apricot fillings of the following formulae were used:

Lemon
- Water—1 qt.
- Sugar—1½ lb.
- Juice and rind of 2 lemons
- Cornstarch—3 oz.
- Salt—⅛ oz.
- Egg yolks—2

Orange
- Water—1 qt.
- Sugar—1½ lb.
- Juice and rind of 2 oranges
- Cornstarch—3 oz.
- Salt—⅛ oz.
- Egg yolks—2

Pineapple
- Water—1 qt.
- Sugar—1 lb.
- Crushed pineapple—2½ lb.
- Cornstarch—3 oz.
- Salt—⅛ oz.

Strawberry—(Formula No. 1)
- Water—1 qt.
- Egg yolks—4
- Powdered whole milk—4 oz.
- Strawberry jam—1½ lb.
- Cornstarch—3 oz.
- Salt—⅛ oz.

Strawberry—(Formula No. 2)
- Water—1 qt.
- Sugar—1½ lb.
- Salt—⅛ oz.
- Fresh strawberries (ground)—1 qt. (dry measure)
- Cornstarch—3 oz.
- Egg yolks—2

Strawberry—(Formula No. 3)
- Water—1 qt.
- Sugar—1½ lb.
- Salt—⅛ oz.
- Powdered whole milk—4 oz.
- Fresh strawberries (ground)—1 qt. (dry measure)
- Cornstarch—3 oz.
- Egg yolks—2

Apricot—(Formula No. 1)
- Water—1 qt.
- Sugar—1 lb.
- Salt—⅛ oz.
- Powdered whole milk—4 oz.
- Canned apricots (ground)—1½ lb.
- Cornstarch—3 oz.
- Egg yolks—2

Apricot—(Formula No. 2)
- Water—1 qt.
- Sugar—1½ lb.
- Salt—⅛ oz.
- Canned apricots (ground)—1½ lb.
- Cornstarch—3 oz.
- Egg yolks—2

A standard custard of the following formula was run with each set of samples in order to have a standard for comparison:
- Water—1 qt.
- Sugar—8 oz.
- Powdered whole milk—4 oz.
- Cornstarch—2½ oz.
- Eggs (whole)—2
- Salt—⅛ oz.

In all cases three-fourths of the water, one-half of the sugar, and all of the powdered milk (where used) were mixed and brought to a boil. To this boiling mixture the fruit (except in the standard custard) was added. The remainder of the sugar was "dry-mixed" with the cornstarch, the eggs (where used) and the salt added, and made into a paste with part of the water. This paste was thinned with the remainder of the one-fourth quart of water and added to the mixture which had been brought to a boil. The completed mixture was boiled for one minute.

In preparing the custards with added lemon juice, varying amounts of lemon juice and rind were added to the standard custard as part of the "thickening" mix. The mixture of lemon juice and rind was prepared by squeezing the juice from the lemons and adding to this juice the grated rinds of all the lemons.

The same strains of *Staphylococcus aureus* and *Salmonella enteritidis* as were used previously by Catheart, Ryberg, and Merz (1942) were used

TABLE 1

Effect of Various Pure Fruit Fillings on Growth of Staphylococcus aureus

1	2	3	4	5
Series	Type of filling	Organisms inoculated per gram of filling	Organisms per gram of filling after 24 hours' incubation	pH
2	Lemon	61,500	1,013	3.10
3		189,420	3,100	3.12
4		201,090	41,000	3.24
5		179,350	2,008	3.09
2	Pineapple	66,340	4,700	3.42
3		159,780	16,450	3.45
4		277,680	244,500	3.62
5		253,300	200,000	3.62
2	Orange	57,830	237,000	3.79
3		174,870	670,000	3.83
4		188,070	870,000	3.82
5		168,920	22,100,000	4.65
2	Strawberry	76,010	162,500,000 [1]	5.00
3		181,760	34,400,000 [3]	4.90
4		274,460	24,500 [2]	3.83
5		297,930	84,000 [2]	3.81
2	Apricot	84,580	495,000,000 [1]	5.43
3		193,100	109,500,000 [1]	5.18
4		188,070	510,000 [2]	4.05
5		170,320	8,850,000 [2]	4.37
2	Custard	80,540	359,500,000	6.73
3		210,570	1,050,000,000	6.77
4		277,680	230,000,000 [4]	6.81
5		241,190	122,500,000 [4]	6.84

[1] Formula No. 1. [2] Formula No. 2. [3] Formula No. 3. [4] Counts of organisms on this custard are doubtful since the custards were heavily contaminated with organisms other than *Staphylococcus aureus* which masked the staphylococci colonies on the blood agar plates.

in this work. Inoculums were made by inoculating tubes of nutrient broth and incubating these tubes for 24 hours at 36.7 to 37.2°C.(98 to 99°F.). Fillings were inoculated with known numbers of organisms and incubated in sterile jars for 24 hours at 98 to 99°F., except for the final two runs with Salmonella in which case the fillings were incubated for 48 hours at the same temperature.

The incubated fillings were stirred and a 25-gram sample of the aggregate taken. This 25-gram sample was then dispersed in 100 milliliters of sterile water by means of an electric stirrer. The samples were then plated by the decimal dilution method. Blood agar was used for plating for the

staphylococci and nutrient agar at first, and later Endo's agar was used for the Salmonella.

Plates were counted both 24 and 48 hours after being plated, but only the 24-hour counts are used since there was no appreciable difference in the counts at the two time intervals.

RESULTS AND DISCUSSION

Data on the effect of pure fruit fillings on the growth of *Staphylococcus aureus* are given (Table 1) and the effect on *Salmonella enteritidis* (Table 2). Column 1 in both tables gives the series number. Each series contains all of the different fillings investigated and the fillings in each series were treated simultaneously. Comparisons of the actual counts must be made on a series basis rather than comparing all the counts together. Since conditions of growth (pH, number of organisms inoculated per gram, etc.) varied from series to series, a direct comparison of the counts on, for example, the lemon filling in Series 2 and the orange filling in Series 4 (Table 1) may lead to erroneous conclusions. The pH values of the fillings are given in the tables.

Because of the low counts on the fruit fillings inoculated with Salmonella no accurate figures can be given in the majority of cases. For this reason, in Column 4 of Table 2 the maximum counts are given; for example, the count on the lemon filling (Series 6) is given as less than 200 per gram. This means that no plate of the 1 to 100 dilution showed more than two colonies.

Little or no effect could be observed upon the addition of lemon juice to standard custard until such high concentration of lemon juice was reached as to give an unsatisfactory flavor (Table 3). When 200 or 300 c.c. of the lemon juice and grated rind mixture were added to a one-quart batch of custard, a decided effect on the growth of *Staphylococcus aureus* could be observed. However, even when 300 c.c. were added the effect was in no way comparable to the effect exhibited by pure lemon fillings on the formula given in this paper. A study of the pH values indicates that there must be some inhibitory factor in the pure fruit fillings other than pH. This may be due to the lack of the food elements of milk in the pure fruit fillings.

The addition of 50 c.c. or less of the lemon juice-rind mixture to one quart batch of standard custard had no harmful effect on the flavor of the custard. When greater amounts were added, the flavor was decidedly acid; actually the flavor was unsatisfactory for a custard.

The results of these experiments show that the pure fruit fillings used are very efficient in retarding the growth of both *Staphylococcus aureus* and *Salmonella enteritidis*. This effect seems to be controlled by both the active acidity of the fruits and the types of acids present in the fruits. The pure lemon filling was found to be most effective in retarding the growth of *Staphylococcus aureus* with the strawberry, pineapple, apricot, and orange fillings following in the order named. In determining this order the strawberry and apricot fillings in which milk was used are disregarded since the addition of milk tremendously reduced their effec-

TABLE 2

Effect of Various Pure Fruit Fillings on Growth of Salmonella enteritidis

1	2	3	4	5
Series[1,2]	Type of filling	Organisms inoculated per gram of filling	Organisms per gram of filling after incubation[3]	pH
6	Lemon	50,000	<200	3.01
7		26,490	<700	3.18
8		105,510	<200	2.92
9		183,350	<200	3.13
6	Pineapple	64,200	<100	3.48
7		23,450	<700	3.62
8		97,500	<500	3.68
9		198,100	<200	3.60
6	Orange	51,800	<1,000	3.80
7		28,860	<1,000	3.86
8		118,570	<3,000	3.80
9		187,390	<500	3.82
6	Strawberry[4]	76,500	<400	4.15
7		38,800	8,350[5]	4.02
8		122,060	<800	4.08
9		209,470	<200	4.08
6	Apricot[4]	60,000	<2,000	4.34
7		29,640	12,700[5]	4.17
8		132,760	<3,000	4.38
9		178,390	<200	4.24
6	Custard	69,100	18,500,000	6.75
7		26,190	2,250,000	6.82
8		112,470	28,250,000	6.62
9		198,100	145,000,000	6.80

[1] Series 6 and 7 of each group were plated on nutrient agar; fillings incubated 24 hours; plates incubated 24 hours. [2] Series 8 and 9 of each group were plated on Endo's agar; fillings incubated 48 hours; plates incubated 24 hours. [3] Figures in this column with the "less than" sign preceding them are maximum counts. For example the <1000 count for Series 6, orange, means that no plate of the 1:1000 dilution had more than one colony. [4] Formula No. 2 was used for the apricot and strawberry fillings. [5] Doubt may be cast on these counts since the fillings were slightly contaminated with an organism other than Salmonella enteritidis which may have been mistaken for the Salmonella in counting the plates.

TABLE 3

Effect of Addition to Standard Custard of a Mixture of Lemon Juice and Lemon Rind on Growth of Staphylococcus aureus

1	2	3	4	5
Series	Lemon juice and rind mixture per quart batch standard custard	Organisms inoculated per gram of filling	Organisms per gram of filling after 24 hours' incubation	pH
	c.c.			
1	0	1,612,000	704,000,000	.....
1	10	1,663,000	752,000,000	.....
1	30	1,520,000	650,000,000	.....
1	50	1,466,000	622,000,000	.....
2	0	17,000	378,400,000	6.79
2	100	15,700	101,600,000	4.51
2	200	12,550	80,600,000	3.95
3	0	173,080	800,000,000	6.71
3	50	151,470	690,000,000	5.38
3	300	118,940	70,000,000	3.64

tiveness. This apparently was due to the buffering action of the milk on the fruit acids.

The effectiveness of these fruit fillings on the growth of *Salmonella enteritidis* apparently falls in the same order, but no list can be made because of the inability to obtain definite counts.

From the tables it can be seen that in general lower pH values give greater inhibition; however, this does not hold true in all cases, as, for example, the strawberry filling (formula No. 2, Table 1) seems to be more effective than does the pineapple filling although the strawberry filling has the higher pH. Also, the apricot filling (formula No. 2, Table 1) appears to be more effective than the orange in spite of their pH relationship. This may be due to the different kinds of acids and/or other substances present in the various fruits.

The results given in this paper hold only for the fillings as specified by the formulae given. Any change in the formula may have a decided effect on the results as shown by the decided decrease in effectiveness of the strawberry and apricot fillings when milk was included in the formula. Also, pure fruits must be used, as a decided difference in effectiveness is indicated between the strawberry filling (formula No. 1, Table 1) using strawberry jam and the filling (formula No. 3, Table 1) using fresh strawberries, both of which contain milk in the same proportions.

SUMMARY

The growth of both *Staphylococcus aureus* and *Salmonella enteritidis* was found to be effectively inhibited in lemon, orange, pineapple, apricot (formula No. 2), and strawberry (formula No. 2) pure fruit fillings of the formulae given in this paper.

This inhibitory action appeared to be greater on the Salmonella organisms than on the staphylococci. The degree of inhibition seemed to be at least partially controlled by the pH of the filling. The entire inhibitory action seemed to be determined by a relationship between the pH and the types of acids and/or other substances present.

The addition of ingredients such as milk, which may exert a buffering action on the fruit acids, reduces the above inhibitory action.

The addition of a mixture of lemon juice and grated lemon rind to a standard custard had no marked effect on the growth of *Staphylococcus aureus* until such high concentrations were reached as to make the flavor of the resultant custard unsatisfactory, and even then the inhibitory action was not great enough to be of much value.

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EFFECT OF PROCESSING ON CAROTENOID (PROVITAMIN A) CONTENT OF PEACHES[1,2]

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The United States commercial fruit-juice pack has increased from about seven million cases in 1938 to about 23 million cases in 1939. In addition to this, facilities for preparation and preservation of frozen products has increased tremendously during this period. These fruit products now form a considerable portion of our diet, and it was believed desirable to investigate the effects of the various processes of manufacture on the nutritional values of such foods. Since the number of different fruits involved would have necessitated an experiment too large for the scope of this work, it was decided to study the effects of processing on the carotenoid (provitamin A) content of peaches.

A review of the literature showed little or no information concerning the effect of fruit-juice processes on the vitamin A content of peaches, and so far as could be ascertained no study has been made on the correlation of carotenoid content of peaches with their vitamin A activity.

Of the many experiments designed to show the heat stability of the carotenoids, one of the earliest was that of Steenbock, Boutwell, Sell, and Gross (1920). Scheunert (1930) and Fellers (1936) stated that cooking of vegetables and fruits will not appreciably influence their vitamin A content. Joslyn (1937) listed some of the factors involved in retaining the vitamin content of fruit juices as choice of fruit, method of extraction, deaeration, metallic contamination, filtration, preservation, packaging, and storing.

Freezing has been shown by Newton (1931) to have no adverse effect on vitamin A potency in peaches. Morgan and Field (1930) found dehydration better than sun-drying for retaining the vitamin A potency of peaches.

REVIEW OF METHODS

Two of the three quantitative procedures available to the investigator wishing to determine the vitamin A content or activity of a specific food were used in this work; namely, a bioassay method and a modified colorimetric method. The colorimetric method used was a modification of the determination used by Zimmerman, Tressler, and Maynard (1940). How-

[1] A portion of a thesis submitted to the Graduate School of the Massachusetts State College in partial fulfillment of the requirement for the degree of Doctor of Philosophy. Agricultural Chemical Research Contribution No. 4.

[2] This work was part of a coöperative project with the New York State Agricultural Experiment Station, Geneva, New York.

[3] Now with New York Agricultural Experiment Station, Geneva, N. Y.

ever, on a theoretical basis alone such a method could not be satisfactory when used with a product such as peaches. Peaches contain not only beta-carotene which may be measured directly but also cryptoxanthin which is only half as potent as beta-carotene. It becomes necessary, therefore, that the two pigments be separated and measured individually.

The presence of cryptoxanthin and beta-carotene in the petroleum ether extract necessitates a type of separation other than diphasic treatment. Zechmeister and Cholnoky (1938), Cook (1936), and Koschara (1936) have compiled the literature on adsorption methods as developed from the original Tswett (1906) technique. Strain (1934) was one of the first workers to show the usefulness of magnesium oxide for adsorption columns. Strain (1934) and MacKinney (1937), using the Tswett adsorption technique with a magnesium oxide column, found the carotenoids in peaches to be 30 to 40 per cent beta-carotene, 30 to 40 per cent cryptoxanthin, and the remainder to be alpha-carotene, lutein, and zeaxanthin.

It was deduced that a combination of the method used by Zimmerman, *et al.* (1940) and a further fractionation with magnesium oxide should theoretically at least give a chemical means of determining the carotenoid content of peaches so that an interpretation in terms of vitamin A potency could be made.

EXPERIMENTAL PROCEDURE

Rochester, South Haven, Crawford, J. H. Hale, and Elberta varieties of peaches used in this investigation were purchased in five-bushel lots from local fruit growers in and around Geneva, New York. Some of the varieties, obtainable in this area only from the New York State Agricultural Experiment Station orchards, were supplied by the Pomology Division in quantities of about eight quarts each. These were the Sunbeam and Morse seedling (non-browning peaches), Oriole, Golden Jubilee, Vaughan, Goldfinch, Eclipse, Hale Haven, Veteran, Viceroy, Valiant, and Ideal.

The fruit received for processing was tree-ripened; any immature or rotted peaches were discarded. The types of products prepared were essentially (1) frozen fruit pulps, (2) frozen sliced peaches, and (3) peach juices.

FROZEN FRUIT PULPS

Frozen fruit pulps were prepared by passing washed, halved, and pitted peaches through a screw-type, force-feed pulper. The machine used in this investigation was a Sep-ro-siv, with stainless-steel bowl, force feeder, and screen. Increasing increments of sucrose were added to portions of the pulp so that concentrations of 10, 20, 25, or 30 per cent, respectively, of the added sugar by weight were obtained. The sugar was dissolved by stirring and the product was packed in No. 1 enamel-lined tin cans and frozen in a brine bath at —23°C.(—10°F.). The cans of pulp were agitated while freezing in order to induce a smooth texture owing to small ice-crystal formation. The frozen pulps used for correlation of the chemical and bioassay methods received the same treatment except that no sugar was added. The peaches prepared for bioassay were from the large lots of peaches: Elberta, J. H. Hale, South Haven, Rochester, and Crawford. The pulp for each bioassay was made from

approximately 25 pounds of peaches (five pounds from each bushel) so that a representative cross section of the variety was used.

Eight-quart batches of the remaining varieties received the treatment outlined above and all samples were stored at —10°F.

FROZEN SLICED PEACHES

The method of preparation of peaches for freezing was that of Woodroof (1930). The fruit was dipped in 10 per cent sodium hydroxide solution at 60°C.(140°F.) for two minutes. The skins were rubbed off by hand, using rubber gloves for protection. The peeled fruit was rinsed in fresh water and then allowed to remain in a two-per cent citric acid solution for about five minutes until sliced. The sliced peaches were covered with a 50-per cent sucrose syrup, placed in parchment paper-lined cartons, and frozen in a Birdseye Multiplate Freezer —32°C.(—25°F.) for two and one-half hours. On removal from the freezer the cartons were wrapped with a waxed paper. The paper was wrapped so that the ends could be folded over and heat-sealed by means of a hot iron to give a moisture-proof package. The packages were stored at —40°C.(—40°F.) until analyzed chemically.

PEACH JUICES

Because of the difference in extraction by a hydraulic press and a Sep-ro-siv, two types of fruit juice were prepared. Hereinafter that type from the hydraulic press will be referred to as "cloudy" and the juice from the Sep-ro-siv will be called "pulpy." Treatment of the peaches for juices was governed to a limited extent by results obtained using the Rochester peach, since it was the first commercial variety to mature.

Cold-pulped juices were prepared essentially by the method given above under the section "Frozen Fruit Pulps." Hot Sep-ro-siv pulped juices differed only in initial treatment in that the fruit was heated to 66°C.(150°F.) before passing through the pulper. The pulpy products were (1) simply pasteurized; (2) deaerated and pasteurized; (3) centrifuged and pasteurized; or (4) centrifuged, deaerated, and pasteurized. Because of the high viscosity of pulpy juices, several of the batches were diluted with one-fourth their weight of a 20-per cent sucrose syrup to facilitate handling.

Cloudy juices were prepared by pressing the fruit in a hydraulic fruit press. The fruit was washed, halved, pitted, put through a crusher, and pressed using a double layer of burlap press cloth. Batches of the fruit were heated to 10°C.(50°F.), 54°C.(130°F.), 66°C.(150°F.), respectively, before being pressed. The pressed or cloudy juices were either immediately pasteurized and bottled or deaerated, pasteurized, and bottled.

Since the batches of juice were rather limited in quantity, they were pasteurized by heating in a stainless-steel, double-jacketed kettle to 77°C. (170°F.) and put in bottles and enamel-lined cans. The containers filled to the maximum and closed were allowed to remain on their sides for three minutes to sterilize the closure and then cooled immediately in cold water to room temperature. The bottled juices were stored at room temperature in the dark until analyzed for vitamin A content.

QUANTITATIVE DETERMINATION OF CAROTENOIDS

In order to determine the validity of the chemical method, frozen-fruit pulps were prepared as stated above and stored at —23°C. (—10°F.). The frozen pulps from Elberta, Crawford, Rochester, South Haven, and J. H. Hale varieties were used in evaluating the vitamin A activity. Samples of these frozen pulps were used for bioassay and at the same time analyzed chemically in order to preclude any possibility of storage effects on vitamin A potency.

TABLE 1

Estimation of Vitamin A Potency of Frozen Peach Pulps From Carotenoid Content (By Chemical Determination)

Peach variety	A—Total carotenoid content (gamma per gram)	B—Beta-carotene content (gamma per gram)	I. U. vitamin A per gram [1]
Rochester	13.5	5.3	13.2
Elberta	14.2	4.6	13.2
J. H. Hale	14.2	4.1	12.8
South Haven	11.6	5.2	11.8
Crawford	20.8	6.7	19.3

[1] I. U. vitamin $A = \left[B + \frac{(A-B)}{2} \right] \times 1.4$.

The bioassay was conducted according to the U. S. P. XI Supplement (1937). A "curve of reference" described by Richardson and Mayfield (1937) was used to determine directly the vitamin A content of a given substance in terms of growth response for a given rat colony.

TABLE 2

Comparison of Results From Bioassay and Chemical Determination for Vitamin A in Frozen Pulps

Variety	Bioassay I. U. vitamin A per gram	Chemical determination I. U. vitamin A per gram	Deviation from bioassay
			pct.
Rochester	16.2	13.2	—18.5
Elberta	11.2	13.2	17.9
J. H. Hale	12.7	12.8	0.8
South Haven	14.0	11.8	—15.7
Crawford	20.4	19.3	—5.4

Since the chemical determination involved the use of photoelectric colorimeter with a Wratten filter No. 47A, it was necessary to standardize the instrument. The standard solution was made by dissolving 6.7 milligrams of pure beta-carotene in 1,000 milliliters of petroleum ether. Several known dilutions of the standard solution were made and each concentration measured on the galvanometer as a deflection point. Deflection points were plotted against gamma of beta carotene per milliliter of the standard solution to give the curve used in the subsequent work.

Twenty to 30-gram samples of the frozen pulp were weighed and immediately placed in a flask containing 100 milliliters of hot diacetone. The size of the sample was governed by the amount of sucrose that had

been added, that is, in every instance an attempt was made to have at least 20 grams of the original pulp in the sample. Since the sugar had been added on a weight basis, the calculation of carotenoid content was computed on the basis of amount of peach originally present in the sample. The analysis consisted of two determinations, one for total carotenoid, Part A, and an empirical measurement of the beta-carotene, Part B, evolved for this experiment. Part A of the analysis was performed according to Zimmerman, *et al.* (1940).

TABLE 3

Comparison of Carotenoid (Provitamin A) Content of Fresh Peach Pulp With That of Frozen Peach Pulp Stored at —23°C.(—10°F.) for Six Months (Chemical Determination)

Variety	Fresh	Stored (frozen)
	I. U. vitamin A per gram	I. U. vitamin A per gram
Rochester	16.7	13.2
Elberta	14.1	13.2
J. H. Hale	12.5	12.8
South Haven	12.3	11.8
Crawford	19.3	19.3

To a suitable volume (usually 100 milliliters) of the total carotenoid solution from Part A, 1.5 to 2.0 grams of magnesium oxide (C. P. Baker's Analyzed) were added, shaken, and filtered through a Jena fritted glass filter (No. 3 porosity) on the filtering surface of which had been placed .2 to .4 gram of magnesium oxide. During the filtration only enough vacuum was applied to draw the solution through the magnesium oxide layer. The receiving vessel used was a Kohlrausch flask in which the filtrate could be made to volume directly without involving any extra transfers. The filtrate, made to volume, was subjected to colorimetric examination; the result interpreted as gamma of beta-carotene.

As the colorimeter standardization was made with pure beta-carotene, any pigment concentration determined in reference to the curve would be read as gamma of beta-carotene per milliliter of solution. For a given sample of peach two readings on the colorimeter, i.e., (A) before and (B) after adsorption, were interpreted as total carotenoids and beta-carotene, respectively. Although .6 gamma of pure beta-carotene is equal to one International Unit of vitamin A—Hume and Chick (1935)—owing to inherent differences of biological response of assay animals, the arbitrary factor 1.4 rather than the theoretical 1.66 is used as an equivalent for one gamma of beta-carotene. However, since the remaining carotenoids, alpha-carotene, gamma-carotene, and cryptoxanthin, are only half as active as beta-carotene molecule for molecule, the difference between (A) and (B) expressed mathematically $\left(\frac{A-B}{2} \times 1.4\right)$ represents their vitamin A potency in International Units. The total vitamin A potency of a sample then becomes $\left[B + \left(\frac{A-B}{2}\right)\right] \times 1.4$. Results of the chemical determination of five varieties of fresh peach conducted concomitant with the bioassay are given (Table 1).

TABLE 4

Effect of Sucrose Concentration on Retention of Vitamin A Potency of Frozen Peach Pulps Stored at —23°C.(—10°F.) (Chemical Determination)

Variety	Sucrose concentration per cent on weight of fruit	Vitamin per gram International Units
Rochester	0	13.1
	10	15.0
	20	15.0
	25	15.0
Elberta	0	13.1
	10	13.0
	20	11.8
	25	11.5
J. H. Hale	0	12.8
	10	12.1
	20	10.3
	25	10.1
South Haven	0	11.8
	10	12.2
Crawford	0	19.3
	10	18.8
	20	18.8
	30	19.2
Sunbeam	25	16.3
Oriole	20	20.9
	25	20.5
Golden Jubilee	20	13.5
	25	13.1
	30	13.5
Vaughan	20	20.1
	25	20.0
	30	21.0
Goldfinch	10	19.3
	20	19.7
Viceroy	20	12.4
Valiant	10	11.0
	20	13.4
Ideal	10	17.4
	20	17.1
Morse	10	17.5
	20	18.0
Eclipse	20	16.5
Hale Haven	0	19.1
	10	19.0
	20	20.0
Veteran	20	15.0

The results of the bioassay and chemical methods are presented for comparison (Table 2). The accepted inherent error for bioassay determinations is said to be about 20 per cent; the results in this table show that the correlation falls within the allowable variation. Since the tendency in vitamin assay at present is to hasten the process without decreasing accuracy, the chemical method as used was deemed acceptable for the purpose of the work.

EFFECT OF VARIOUS PROCESSES ON PROVITAMIN A CONTENT

The provitamin A content of fresh samples of Rochester, South Haven, Elberta, J. H. Hale, and Crawford peaches was determined using the chemical method. In addition to determinations of carotenoid (provitamin A) content of the frozen pulps of the above-mentioned varieties, estimation of carotenoid content of Sunbeam, Oriole, Golden Jubilee, Vaughan, Goldfinch, Viceroy, Valiant, Ideal, St. John, Eclipse, Hale Haven, Veteran, and Morse varieties was made in order to have a relative index of vitamin A potency.

TABLE 5

Effect of Peeling and Freezing on Carotenoid (Provitamin A)[1] Content of Sliced Frozen Peaches (Chemical Determination)

Variety	Fresh slices unpeeled	Fresh slices peeled	Frozen slices
Rochester	26.5	15.3	15.3
Elberta	19.6	13.6	14.0
J. H. Hale	17.8	13.9	13.4
South Haven	16.7	14.6	14.4
Crawford	22.5	17.4	17.3

[1] International Units of vitamin A per gram.

A comparison of the vitamin A content of the freshly prepared pulp for freezing with that of the frozen product stored at —23°C.(—10°F.) for approximately six months (Table 3) shows that within the experimental error there was no appreciable loss of provitamin A content in the frozen products owing to storage.

A record of the effect of increasing concentrations of sucrose on the retention of vitamin A potency of frozen peach pulps stored at least six months at —23°C.(—10°F.) (Table 4) indicates that sucrose is not necessary for protective action at this low temperature. The results also serve as an index of the relative provitamin A content of the unsweetened pulps made from the several varieties used. It will be noticed in this table that several varieties show no zero concentrations of added sugar. However, since the varieties having the controls showed no significant changes in vitamin A content on storage and since the computations were based on sugar-free pulps, it was decided to include these peach varieties having no controls.

Results showing the effects of peeling and freezing on the carotenoid (provitamin A) content of sliced frozen peaches (Table 5) show that although the peeling resulted in an average loss of approximately 25 per

cent in provitamin A content, the freezing storage showed no additional loss.

The results of a study to determine the effects of the individual fruit-juice processes on loss of provitamin A from peach juice are presented (Table 6). Cold pulping of the fruit showed the least loss of vitamin A precursors during the preparation process. Centrifuging of the juice resulted in a net loss averaging 20 per cent. Deaeration showed a conservation of provitamin A content of about 10 per cent over those samples which were centrifuged and pasteurized rather than centrifuged, deaerated, and then pasteurized.

TABLE 6

Effect of Processing on Carotenoid (Provitamin A) Content of Peach Juices (Chemical Determination)

Processes involved	Loss of provitamin A (based on fresh peach)
	pct.
Cold pulped at 10°C.(50°F.)	28
Centrifuged 50°F	74
Centrifuged, pasteurized	81
Centrifuged, deaerated, pasteurized	81
Hot pulped at 66°C.(150°F.)	45
Centrifuged 150°F	59
Deaerated, pasteurized	53
Centrifuged, pasteurized	89
Centrifuged, deaerated, pasteurized	80
Three parts pulp, one part 20-per cent syrup, deaerated, pasteurized	72
Cold pressed at 50°F	71
Centrifuged 50°F	89
Centrifuged, deaerated, pasteurized	95
Pasteurized	78
Hot pressed at 150°F	71
Centrifuged 150°F	85
Centrifuged, pasteurized	90
Pasteurized	81
Deaerated, pasteurized	80
Centrifuged, deaerated, pasteurized	85
Three parts pulp, one part 15-per cent syrup, deaerated, pasteurized	90

SUMMARY

A chemical method to determine the vitamin A content of a peach sample containing carotenoids of varying vitamin A potency was developed by combining a beta-carotene determination and a Tswett adsorption column technique.

Animal bioassay values were shown to agree within experimental limits with chemical evaluation of the vitamin A content of frozen pulps from peaches of the Rochester, Crawford, South Haven, J. H. Hale, and Elberta varieties.

The potential vitamin A contents for fresh peaches of Rochester, J. H. Hale, Crawford, South Haven, and Elberta varieties were found to be

13, 13, 12, 12, and 19 I. U. per gram, respectively, as determined by the chemical method and confirmed by bioassay.

Frozen sliced peaches and frozen peach pulps of the above varieties were shown to contain approximately 75 per cent of the vitamin A potentially available in the whole fresh peach.

Chemical determination of vitamin A precursors before and after six months of freezing storage at —23°C.(—10°F.) showed no significant changes in pulps made from Rochester, J. H. Hale, Crawford, South Haven, and Elberta varieties.

Studies of frozen pulps from several varieties of peaches sweetened with sucrose showed no significant variation in retention of the vitamin A potency over a storage period of six months at —23°C.(—10°F.).

Pressing of peaches for juice resulted in losses of approximately 50 per cent of the carotenoids possessing vitamin A activity while centrifuging of these peach juices resulted in additional losses of vitamin A precursors up to 30 per cent.

The deaeration and pasteurization processes as such showed no special effect on the retention of provitamin A content

Pulpy juices prepared by cold-pulp extraction, diluted with a sugar syrup, and deaerated gave a commercially feasible juice although low in provitamin A.

In conclusion, it would seem from a study of provitamin A content that either frozen sliced peaches or frozen peach pulp is of greater nutritive value than peach juices. Losses ranging from 50 to 90 per cent of the vitamin A potency of the fresh fruit seem to indicate a necessity for improved methods of production and preservation of fruit juices.

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PROTEIN AND SULFUR CONTENTS OF IMMATURE LIMA BEANS *(PHASEOLUS LUNATUS)* AS AFFECTED BY VARIETAL AND ENVIRONMENTAL FACTORS AND PROCESSING

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The data presented herein are part of the results of studies made during the last two and one-half years as a joint research by the United States Department of Agriculture and the University of California.[1] The present report is concerned with the protein and sulfur contents of green lima beans as affected by hereditary and environmental factors.

Interest in immature or so-called fresh lima beans has been stimulated by the discovery that they produce an excellent frozen-pack product. Hitherto the fresh market season has been short and local in character, but the new processing has placed these products in the year-around market, and thus they have become important items in the American dietary. As a result of experimental studies for quality in the frozen-pack, the authors found themselves in possession of a group of samples that offered unusual opportunities for a study of variations of nutritive values. There were available more than 200 frozen and unprocessed immature seed samples. The genetic history, the environment during production, and the treatment of the stored samples were known, thus consideration could be given to the composition of each type of seed and of the varieties or strains of seeds included in each type, in addition to the influence of soil, climate, maturity, and processing on this composition.

REVIEW OF THE LITERATURE

A review of the literature has shown but meager data on either dry or immature fresh green beans. Researches on the total sulfur or the sulfur distribution in immature beans have not come to the attention of the authors. Sherman (1937) stated, "Minerals are undoubtedly present (in fresh limas) but quantitative data have not been found." Furthermore, there are few citations covering total nitrogen or the nitrogenous constituents of these beans. Carolus (1931) reported a maximum range in nitrogen of from 1.26 to 1.59 per cent in samples of the small flat Henderson Bush beans stored in pods for 24 and 48 hours at normal

[1] Acknowledgment is made of the assistance of Professor W. W. Mackie, Department of Agronomy of the University of California, in supervising the horticultural side of the project, and to D. G. Sorber, of the Bureau of Agricultural Chemistry and Engineering, for assistance with the technological problems. Later publications will deal directly with their part of the project.

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temperature. The samples stored for 24 hours contained the higher concentration of nitrogen. Recalculated as protein these samples varied from 7.88 to 9.94 per cent. The nitrogen occurring in other samples which were stored in a refrigerator for 24 and 48 hours was found to be 1.35 and 1.34 per cent. This author also reported soluble nitrogen ranging from .03 to .19 per cent of the total weight of moist beans. Again the higher percentage was found in the sample stored 24 hours at normal temperature while the lowest occurred in the sample stored 72 hours also at normal temperatures. Chatfield and Adams (1931) noted five analyses, in their summary on the composition of green vegetables, of green shelled limas *(Phaseolus lunatus macrocorpus)* in which the total protein ranged from 6.4 to 9.4 per cent, while the water content varied from 58.9 to 71.8 per cent. Vitamins B_1 and B_2 have been evaluated by Rose and Phipard (1937) and more recently by Fellers, Esselen, and Fitzgerald (1940). Their data showed an appreciable vitamin content, but the weight of the fraction of nitrogen thus accounted for would be small indeed. According to Serrano (1923), green cultivated limas contain .011 per cent of hydrocyanic acid.

Apparently no studies have been made of progressive changes in composition of the lima-bean solids as they mature. However, in the extensive literature which has accumulated on the composition of dry beans of other species, a few samples of dry lima beans have been studied. Hendry, Jaffa, and Albro (1918) examined 16 bean species including one sample of large lima bean from the Lewis stock and one sample of the small or Henderson Bush variety. The large bean sample contained 11.82 per cent water and 19.25 per cent protein, and while the small one had only 7.14 per cent water, the protein was 19.78 per cent. The range in protein for the entire 16 species was from 18.80 to 26.34 per cent. McHargue and Ray (1931), in their composition studies on dry beans, included one sample of lima-bean seed which they purchased in a grocery store. No description of the type of the seed was given and apparently no account was taken of water content. They found a total protein of 26.44 per cent and a total sulfur of .227 per cent. Jones, Gersdorff, Johns, and Fink (1922), in their study of amino acids in lima-bean proteins on two strains of the large potato type or the Fordhook bean, reported that the total protein in these limas averaged 21.17 per cent. Also these workers found that the natural salts in dry lima beans were sufficient to bring about extraction of a large percentage of the albumins and globulins present. Cystine sulfur was found in both proteins. Harwood, Cole, and Prowse (1934) reported that the protein varied in four varieties of dry beans, not limas, from 20.3 to 27.9 per cent. Greenwood (1933) found that New Mexico Pinto and Bayo beans varied in protein from 20.9 to 29.5 per cent and in moisture content from 8.9 to 9 per cent. Greenwood concluded that there was little difference in the average composition of beans from different portions of the state and moreover that there was an inverse ratio between the total nitrogen and the total yield expressed as pounds per acre of shelled dry beans.

According to these records green or white immature lima beans range in protein from 6.40 to 9.94 per cent. They contain water-soluble nitro-

gen, some of which occurs in nonprotein molecules. They contain sulfur as cystine and in vitamin B_1. Dry lima beans, on the contrary, range from 19.25 to 26.44 per cent protein, and while sulfur is present, the cystine sulfur is relatively low.

EXPERIMENTAL PROCEDURE

The samples studied were grown in eight experimental plots set aside for this purpose. The precise location of the plots was determined largely by expediency, although a serious attempt was made to secure locations in which lima beans were a normal and important crop. These plots were located from Rio Hondo Farm in the southern part of California to San Luis Obispo in the central section. Soil, prevailing temperature, and other climatic conditions, therefore, are representative of growing conditions in the southern half of the state. In general the plots fall into three climatic groups: (1) coastal valley plots at San Luis Obispo and Santa Maria, both of which enjoy moderately cool temperatures with considerable fog during the growing season; (2) inland valley locations at Whittier and San Fernando where there is less fog together with long, sunny, hot days during the growing season; and (3) locations at Milpitas, Camarillo, Rio Hondo, and Hawthorne which have moderately warm temperatures with adequate sun and some fog. All of these plots were under the constant supervision of an expert farm manager or a trained agent such as the county agricultural extension agent.

The seeds were selected from varieties of lima beans whose genetic history was known. Selections were made from the four recognized morphological types, namely, the large flat, the large thick or large potato, the small flat, and the small thick or small potato types.

Three groups of samples are included in this report. Group I, the frozen mixed 1937, samples were packed in the year 1937. These were random samples from the plots planted that year. They were all blanched and cooled in tap water and frozen in a current of air on screens within 10 to 12 hours after picking. These samples were not uniformly green but contained various percentages of white or almost white beans. Group II, the frozen green 1938, samples were grown in the year 1938. They were blanched and cooled in tap water and sorted into white, or partly white, and uniformly green beans before being placed in the freezer. The green and white beans were frozen and stored as two separate samples. Analysis has not been made on the white beans to date. Group III, the unprocessed mixed 1938, samples were random aliquot samples taken from the batches of the 1938 crop before shelling. Each aliquot represented 20 per cent of the total weight of pods in a harvest. The samples were shelled, cut, dried, ground, and stored in air-tight bottles in a cool, dark place until needed for analysis. Samples of all groups were in storage from eight weeks to 14 months before analytical work was completed.

In order to reduce to a minimum the danger of variations owing to the relative portions of white and green beans in any sample, a relatively large portion of 100 to 150 grams of beans was ground and mixed. Nitrogen and water in the frozen samples were determined on freshly ground material. Sulfur determinations were made on desiccated ground prep-

TABLE 1

Protein, Water, and Sulfur as Related to Seed Strains and Methods of Processing

(Per cent on weight of beans)

Type of bean	Strain code [1]	Group I—frozen mixed 1937						Group II—frozen green 1938						Group III—unprocessed mixed 1938					
		Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur
			pct.		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*
Large flat	7402	5	6.11	5	76.08	5	.023	2	5.48	2	76.14	2	.021	....	.....	1	80.37	1	.022
	7404	4	5.50	5	77.80	5	.019	3	5.92	3	75.79	3	.015	2	6.75	2	71.88	2	.022
	W.I.B.	3	5.19	3	78.60	3	.018	2	6.15	2	75.17	2	.020	....	.....	1	71.29	1	.022
	Misc.	13	5.49	13	74.56	....	.....	16	6.14	16	73.81	1	.018	9	7.30	10	72.42	3	.022
Mean	All	25	5.58	26	75.94	13	.021	23	6.01	23	74.39	8	.018	11	7.20	14	72.83	7	.022
Large thick	5552	1	5.80	1	72.47	1	.021	3	6.65	3	72.15	3	.019	2	6.57	3	73.36	3	.025
	H.P.F.	1	5.79	1	74.94	1	.021	2	5.54	2	79.05	2	.018	1	6.93	2	79.12	1	.021
	37799	1	6.71	1	72.18	1	.029	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	C.F.H.	....	.....	....	.....	....	.....	7	6.77	7	71.15	3	.025	1	6.94	1	70.80	1	.028
	Misc.	2	7.43	2	70.44	....	.....	13	6.19	13	72.67	....	.....	5	7.35	3	67.83	1	.023
Mean	All	5	6.63	5	72.09	3	.024	25	6.36	25	72.69	8	.022	9	7.08	9	72.94	6	.025
Small flat	5860	2	6.97	2	66.75	2	.026	2	7.48	2	71.30	2	.024	1	6.13	2	73.36	2	.028
	C.H.B.	7	8.15	1	67.81	1	.026	2	7.78	2	65.20	....	.....	....	.....	....	.....	....	.....
	6018	3	7.78	5	66.14	4	.031	3	7.30	3	70.01	2	.025	3	7.87	3	65.09	3	.031
	Misc.	24	7.00	24	69.43	....	.....	25	7.13	25	70.71	....	.....	17	7.58	18	68.34	3	.022
Mean	All	36	7.29	32	68.70	7	.029	32	7.57	32	70.34	4	.024	21	7.55	23	68.35	8	.027
Small thick	37701	1	7.40	1	70.34	1	.022	2	7.06	2	69.76	2	.026	1	6.75	1	65.35	1	.030
	37722	1	7.09	1	69.17	1	.023	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	8188	....	.....	....	.....	....	.....	2	6.71	2	72.22	2	.023	2	6.90	2	76.02	2	.024
	Misc.	3	7.67	3	69.49	....	.....	15	6.93	15	69.23	....	.....	4	6.85	4	67.17	....	.....
Mean	All	5	7.50	5	69.60	2	.023	19	6.92	19	69.60	4	.025	7	6.85	7	69.44	3	.026

[1] Strain code—numbers refer to Professor Mackie's strains. W.I.B. = Wilson's Improved Bush; H.P.F. = Hagen Pole Fordhook; C.H.B. = Commercial Henderson Bush; C.F.H. = Commercial Fordhook (Hawthorne).

arations. Official methods of analysis (1935) were used in all cases. The nitrogen was multiplied by 6.25 for protein. Sulfate was determined gravimetrically on ash that had been treated with magnesium carbonate as well as on untreated ash. These sulfur data will be discussed in detail elsewhere, but it should be noted that statistical analysis showed them to be identical with reference to their sulfur content; therefore the means reported here include the duplicate tests on both treated and untreated material. Total moisture was found by drying in a vacuum oven at 70°C. (158°F.) to constant weight.

All data have been calculated as per cent of the moist whole bean and as per cent of the total bean solids. The significance of the variations found was determined after Snedecor's (1938) "ratio (F)" or as correlation coefficients which were calculated in the usual manner.

TYPE AND VARIETAL OR STRAIN VARIATIONS IN PROTEIN AND WATER IN MOIST BEANS

Calculated as per cent of moist bean, the total protein ranged from 4.44 per cent for the large, flat, frozen sample of bean No. 7426 grown at Santa Maria in 1937 to 10.29 in the small, flat, unprocessed Hopi variety 155 grown at Rio Hondo in 1938. In all, 218 samples were analyzed. These were distributed somewhat unevenly among the eight plots and four types of seed. The mean protein content was 6.65 per cent for the 170 frozen samples and 7.27 per cent for the 48 unprocessed ones. The total range from 4.44 to 10.29 per cent is large but it is neither surprising nor unusual in the field of food composition, according to Sherman (1937) and Chatfield and Adams (1931).

The data were arranged to show the composition of the four types of beans as well as that of the individual seed strains which were included in each type (Table 1). When these data, in each group, were calculated separately for the "ratio (F)," the variances between the strains were not significantly larger than the variance within the strains; for example, there was a range from 5.03 to 7.81 per cent in the five tests on the large, flat bean seed No. 7402, while the range of the four tests on the seed No. 7404 was from 5.00 to 5.92 only. Other types showed similar degrees of strain uniformity. However, when the variances between the means for each type in Groups I and II of the blanched frozen samples were compared with the means for the individual seed within the type, a highly significant variation between types was found. The means of 7.29 and 7.57 per cent protein for the small, flat type in Groups I and II, respectively, were significantly larger than the means of 5.58, 6.63, 6.01, and 6.36 for the large types, and of 6.92 for the small, thick, frozen beans in 1938. The mean for the small, thick type in 1937 was 7.5 per cent. The last five samples mentioned were grown in one plot and were overmature, completely white beans with a relatively low water content. The variation between the means of 7.20, 7.08, 7.55, and 6.85 for types in the unprocessed beans, Group III, was not significant, although the mean of 7.55 per cent for the small, flat type was again slightly higher than the means for the other types.

TABLE 2

Protein, Water, and Sulfur in Lima Beans Grown in Eight Localities

(Per cent on weight of beans)

Type of bean	Plot location [1]	Group I—frozen mixed 1937						Group II—frozen green 1938						Group III—unprocessed mixed 1938					
		Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Water	Number of tests	Sulfur
			pct.		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*
Large flat	C.	8	6.19	8	73.66	3	.022	8	6.19	8	72.96	2	.019	6	6.89	7	72.04	4	.021
	W.	13	5.48	13	77.74	9	.020	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	S.M.	4	4.66	5	74.91	1	.022	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	....	.....	7	6.57	7	75.41	3	.018	5	7.56	7	73.62	3	.023
	S.L.O.	....	.....	....	.....	....	.....	8	5.46	8	75.15	3	.018	....	.....	....	.....	....	.....
Large thick	C.	3	6.88	3	71.11	1	.021	8	6.35	5	72.96	2	.019	4	6.58	5	70.01	5	.025
	W.	1	5.79	1	74.94	1	.021	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	....	.....	5	6.16	3	77.62	3	.020	4	7.62	3	78.53	2	.023
	S.L.O.	....	.....	....	.....	....	.....	3	5.70	1	73.35	....	.....	....	.....	....	.....	....	.....
	H.	....	.....	....	.....	....	.....	7	6.77	7	71.15	3	.025	1	6.94	1	70.80	1	.028
	M.	1	6.71	1	72.18	1	.029	2	6.42	2	72.38	....	.....	....	.....	....	.....	....	.....
Small flat	C.	16	7.01	16	67.81	4	.029	12	7.14	12	69.92	1	.025	10	7.03	11	68.03	4	.023
	W.	2	6.40	2	71.87	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	....	.....	9	7.67	9	70.45	1	.022	9	8.48	10	67.65	3	.031
	S.L.O.	4	6.11	5	69.40	1	.029	9	6.52	9	71.93	2	.025	2	5.93	2	73.57	1	.031
	S.F.	8	8.09	9	69.19	2	.028	....	.....	....	.....	....	.....	....	.....	....	.....	....	.....
	M.	6	7.21	6	64.77	....	.....	2	7.83	2	65.20	....	.....	....	.....	....	.....	....	.....
Small thick	C.	....	.....	....	.....	....	.....	7	6.83	7	69.50	2	.026	5	6.83	5	66.80	1	.030
	R.H.	....	.....	....	.....	....	.....	1	7.00	1	74.11	1	.020	2	6.90	2	76.02	2	.024
	S.L.O.	....	.....	....	.....	....	.....	1	6.42	1	70.33	1	.025	....	.....	....	.....	....	.....
	M.	5	7.50	5	69.20	2	.023	10	7.03	10	69.15	....	.....	....	.....	....	.....	....	.....

[1] Plot locations: C = Camarillo; W = Whittier; S.M. = Santa Maria; R.H. = Rio Hondo; S.L.O. = San Luis Obispo; H = Hawthorne; M. = Milpitas; S.F. = San Fernando.

It is known from records kept at the time of processing that the beans in all types took up variable amounts of water during blanching. Some of this water was undoubtedly on the surface of the individual beans and was lost during the freezing process. However, the small types, especially the small, flat type, contained less water even as unprocessed green beans than did the large types, and from results of studies on cooking quality, to be published later, it was evident that they were definitely slower in the rate at which they imbibed water.

The data show that in all samples the total water content of the moist beans in both the blanched, frozen and the freshly harvested, unprocessed samples is inversely proportional to the protein content (Tables 1 and 2). The coefficient of correlation between protein and water in the 170 frozen samples was minus .59, while the corresponding coefficient in the 48 unprocessed samples was minus .72. These highly significant correlations will be discussed in greater detail in connection with the composition of the beans from different plots. It seemed, however, that such varietal or strain variations as occurred were related to water balance in the type rather than to inherent type or strain variations in the ratio of protein to total solids, and that because the small types were lower in water they appeared to be higher in protein, even as green, immature beans, than were the large types.

PLOT VARIATIONS IN PROTEIN AND WATER IN MOIST BEANS

Since levels of protein apparently were not related specifically to strain or type, it was necessary to look further for the sources of the variations found in this nutrient. The simplest explanation was assumed to be differences in soil and climate according to plot location. The data were therefore rearranged by plot and type (Table 2). Here a plot variation that occurred in all types of beans was clearly demonstrated. When the "ratios (F)" were calculated, the variations between the plots were found to be highly significant in all three groups of samples. Thus the means of the protein per cent in the large, flat type ranged from 4.66 for the Santa Maria plot to 6.19 for the Camarillo plot in Group I, from 5.46 for the San Luis Obispo plot to 6.57 for the Rio Hondo plot in Group II, and from 6.89 for the Camarillo plot to 7.56 for the Rio Hondo plot in Group III. The large, thick type showed plot variations in protein per cent of 5.79 to 6.88, 5.70 to 6.77, and 6.58 to 7.62 in the three groups. The small, flat type, while maintaining a slightly higher protein content in all plots, showed plot variations in protein per cent of 6.11 to 8.09 in Group I, 6.52 to 7.83 in Group II, and 5.93 to 8.48 in Group III. The plot distribution for the small, thick type was poor, but the same tendency was evident in the data available.

The data collected in this experiment referable to yield were limited and inconclusive if not contradictory. The Rio Hondo plot showed a higher protein and higher water content for both the "frozen green" and the "unprocessed" samples than did the Camarillo plot. For example, the large, flat, "frozen green" beans from Camarillo averaged 6.19 per cent protein and 72.96 per cent water, while the corresponding averages for Rio Hondo beans were 6.57 per cent protein and 75.41 per cent

water. The averages in the "unprocessed" samples from Camarillo for the large, flat type were 6.89 per cent protein and 72.04 per cent water, but for Rio Hondo beans they were 7.56 per cent protein and 73.62 per cent water. Nevertheless the Rio Hondo beans were larger, averaging 1.93 grams per bean, than were the Camarillo beans, which averaged only 1.42 grams in this type. At Rio Hondo 35.91 per cent of the shelled crop was white, while at Camarillo only 25.08 per cent had turned white. The relative weight of the shellable pod hulls at Rio Hondo was high, giving a shrinkage at shelling of 64.18 per cent as against 60.55 per cent shrinkage at Camarillo. Thus the Rio Hondo samples had larger beans with higher protein and higher water content, heavier pods, and many white beans as compared with Camarillo. Other types showed similar relationships in the two plots. These decidedly incomplete data on yield indicated that a heavy crop was correlated with a high protein content. This is contrary to Greenwood's conclusions concerning yield of Pinto and Bayo beans, and should be looked upon with open-mindedness until other studies now under way in our laboratories are completed.

SULFUR VARIATIONS AND PROTEIN-SULFUR CORRELATION

Unfortunately it was not possible to carry through as extensive a group of analyses for sulfur as was possible for protein. Only 75 individual samples were examined. With reference to type and strain, these samples were distributed among the eight plots in an irregular manner. First, attention should be given to the range in total sulfur, since it is relatively greater than is indicated in the tables in the literature. The individual samples ranged from .013 to .033 per cent sulfur in Group I, from .012 to .026 per cent in Group II, and from .016 to .037 per cent in Group III. Again when these data were arranged as in Table 1 to show the type and strain variation, it was found that the sulfur varied in precisely the same manner as did the protein. Because of the variation within the seed strains there was not an appreciable variation between the seeds of one type. Nevertheless, the small, flat type was significantly higher in sulfur than the others, the mean per cent being .029, .024, and .027 in the three groups as against corresponding values of .021, .018, and .022 for the large, flat type. The large and small, thick types were intermediary in respect to this element (Table 1). The positive coefficients of correlation between protein and sulfur were .59 for the 46 frozen, paired samples and .81 for the 17 unprocessed, paired samples. These highly significant ratios of correlation suggested strongly that the sulfur was associated with nitrogen, either in proteins or in some other molecule containing both of these elements, although they do not eliminate entirely the possibility that either nitrogen or sulfur may occur in independent forms.

VARIATIONS IN PROTEIN AND SULFUR CONTENTS WITH PROCESSING

Since the nonprotein, nitrogen-containing molecules reported to occur in lima beans—Carolus (1931), Fellers, Esselen, and Fitzgerald (1940), Jones, Gersdorff, Johns, and Fink (1922), Rose and Phipard (1937), and Serrano (1923)—are highly soluble in water, and since Jones, *et al.* (1922) found that certain fractions of the protein nitrogen may be extracted

without additional salt in the solution, a loss of protein nitrogen in any water-blanching process might be predicted. Likewise, one might expect a loss of sulfur in such a process because water-soluble sulfur occurs in the vitamin B_1 molecule in these beans, and traces of soluble sulfur may be present in other unidentified forms. This experiment included both blanched, frozen and unprocessed, matched samples; hence it was possible to consider the losses in blanching and other processing procedures. There were three probable sources of variations with respect to processing—sorting for color, blanching, and storage—that were independent of sampling errors.

A comparison of the plot means of protein per cent for the frozen, mixed 1937 group and the corresponding means for the same plots for the frozen, green 1938 group showed relatively small differences in the two groups. For example, for Camarillo plots the means of protein per cent for the large, flat type were 6.19 in both frozen groups; for the large, thick type they were 6.88 and 6.35; and for the small, flat type they were 7.01 and 7.14, respectively, for these two groups (Table 2). Apparently the removal of white or nearly white beans from the 1938 crops before freezing, instead of freezing green and white beans mixed as in 1937, did not materially affect the protein content of the frozen samples.

From the 1938 crops there were available 45 matched pairs of frozen, green and unprocessed, mixed samples on which nitrogen was determined. The mean protein per cent was 6.93 on the frozen and 7.34 on the unprocessed samples. This difference was not significant but it did seem to indicate that some soluble nitrogen had been lost in the blanching. There was, however, some evidence that this was partly an illusion created by a gain in water in blanching and by the presence of a few prematurely dried, white beans in the unprocessed samples. Also there was a higher-than-usual experimental error here, since in the case of the frozen beans the nitrogen determinations were made on undried samples weighed within a few minutes after removal from frozen storage, whereas in the case of the unprocessed beans the nitrogen determinations were made on samples that had been dried, ground, and stored for various periods before analysis.

There were 15 matched pairs of frozen and unprocessed samples on which sulfur determinations were made. The mean sulfur per cents were .021 and .025 on frozen and unprocessed beans, respectively, and even though there were great variations within each group, the ratio of the variance here was significant. This would be of greater interest and importance from a nutritive point of view if a corresponding variation had occurred in the per cent of sulfur in the total solids. From evidence cited below, it appeared again that this variation was created by differences in the relative water content of the matched samples.

Summarizing the above discussions, it was found that the varieties and strains within each morphological type showed excellent uniformity in their content of total proteins and total sulfur. These totals were decreased slightly by sorting and blanching. Apparent difference between morphological types as regards protein and sulfur contents appeared to be related

TABLE 3

Protein and Sulfur in Bean Solids

(Per cent on weight of solids)

Type of bean	Plot location [1]	Group I—frozen mixed 1937				Group II—frozen green 1938				Group III—unprocessed mixed 1938			
		Number of tests	Protein	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Sulfur	Number of tests	Protein	Number of tests	Sulfur
			pct.		*pct.*		*pct.*		*pct.*		*pct.*		*pct.*
Large flat	C.	8	23.51	3	.083	8	22.85	2	.072	6	22.44	4	.080
	W.	13	23.75	9	.088	.....	.....	....	.....	....	.....	....	.....
	S.M.	4	17.98	1	.097	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	7	26.77	3	.074	5	27.75	3	.085
	S.L.O.	....	.....	....	.....	8	22.05	3	.079	....	.....	....	.....
Large thick	C.	3	23.68	1	.074	8	22.38	2	.064	4	21.26	5	.083
	W.	1	23.10	1	.085	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	5	26.18	3	.083	4	26.37	2	.084
	S.L.O.	....	.....	....	.....	3	21.58	....	.....	....	.....	....	.....
	H.	....	.....	....	.....	7	23.47	3	.076	1	23.66	1	.097
	M.	1	24.13	1	.109	2	23.24	....	.....	....	.....	....	.....
Small flat	C.	16	21.77	4	.084	12	23.68	1	.078	10	22.21	4	.072
	W.	2	22.76	....	.....	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	9	26.64	1	.080	9	26.32	3	.100
	S.L.O.	4	20.10	1	.093	9	23.19	2	.084	2	22.46	1	.114
	S.F.	8	26.40	2	.076	....	.....	....	.....	....	.....	....	.....
	M.	6	22.89	....	.....	2	22.35	....	.....	....	.....	....	.....
Small thick	C.	....	.....	....	.....	7	22.93	2	.084	5	20.62	1	.086
	R.H.	....	.....	....	.....	1	27.04	1	.078	2	29.23	2	.100
	S.L.O.	....	.....	....	.....	1	21.65	1	.083	....	.....	....	.....
	M.	5	25.70	2	.074	10	22.88	....	.....	....	.....	....	.....
All types	C.	27	22.50	8	.083	35	23.61	7	.074	25	21.79	14	.078
	W.	16	24.40	10	.087	....	.....	....	.....	....	.....	....	.....
	S.M.	4	17.98	1	.097	....	.....	....	.....	....	.....	....	.....
	R.H.	....	.....	....	.....	22	26.60	8	.080	20	27.48	10	.092
	S.L.O.	4	20.10	1	.084	21	22.47	6	.078	2	22.46	1	.114
	H.	....	.....	....	.....	7	23.47	3	.076	1	23.66	1	.097
	S.F.	8	26.40	2	.076	....	.....	....	.....	....	.....	....	.....
	M.	12	24.16	3	.084	14	22.85	....	.....	....	.....	....	.....
Average	All plots	71	23.25	25	.085	99	24.02	24	.077	48	24.23	26	.086

[1] Plot locations: C = Camarillo; W = Whittier; S. M. = Santa Maria; R. H. = Rio Hondo; S. L. O. = San Luis Obispo; H = Hawthorne; M = Milpitas; S. F. = San Fernando.

to water content, but a consistent plot variation independent of water content occurred in all types, in all strains, and in all three groups of samples.

VARIATIONS IN PER CENT OF PROTEIN AND SULFUR IN TOTAL BEAN SOLIDS

Obviously, if the above-mentioned variations were valid, they would have been emphasized when the distribution of sulfur and protein in the bean solids were studied. Therefore it was interesting to calculate the composition of the total bean solids with reference to protein and sulfur (Table 3).

In general, the variations found were the same as those discussed above. There were no significant variations between the seeds within a type. The average protein per cent for individual samples in the large, flat type ranged from 17.38 to 28.59 in Group I, from 20.40 to 28.63 in Group II, and from 20.38 to 28.29 in Group III. Similar values for the large, thick type ranged from 21.07 to 27.55, 19.51 to 28.16, and 17.81 to 28.77 in the three groups, respectively. The small, flat type showed ranges of 18.67 to 27.79, 21.84 to 28.11, and 20.22 to 28.96; while the small, thick types showed ranges of 23.00 to 28.84, 19.79 to 27.04, and 19.11 to 31.38 for the three groups, respectively.

There was no significant difference in protein content of total solids between the large and small types of beans. This fact supports the conclusion drawn above that the apparent difference between types observed in the moist beans was essentially a matter of water balance in the beans. The smaller beans, especially the small, flat type, were drier throughout the growing period than the larger types. The plot variation again was highly significant. The lowest protein content occurred in the large, flat beans grown at Santa Maria in Group I, the average being 17.98 per cent. The beans of all types grown at Camarillo, Whittier, San Luis Obispo, and Milpitas were slightly higher in protein, the total range being from 20.10 to 25.70 per cent in the four types and three groups of samples. The highest protein content occurred in the small, flat type at San Fernando in Group I and in all types at Rio Hondo in Groups II and III. The protein in the former plot averaged 26.40 per cent, while in the latter, the averages ranged from 26.18 per cent in the large, thick type in Group II to 29.23 per cent in the small, thick type in Group III. The sulfur varied in about the same manner as the protein and the sulfur-protein correlation again was highly significant in all groups. When all the frozen and unprocessed beans from the 1938 crop were compared, the average protein content was 24.02 and 24.23 per cent, while the mean sulfur content was .077 and .086 per cent, respectively. In neither instance were these differences significant.

SUMMARY

Studies have been completed on the protein composition of 170 samples of frozen lima beans and 48 samples of unprocessed beans. In addition, 75 samples have been analyzed for total sulfur.

The protein content of the frozen beans averaged 6.65 per cent, while that of the unprocessed beans averaged 7.27 per cent.

The total sulfur content of the 49 samples of frozen beans analyzed averaged .022 per cent, while that of the 24 unprocessed samples averaged .026 per cent.

The significant variations in protein and sulfur contents found in moist beans of different morphological types were related to water content rather than to a fundamental difference in the distribution of protein and sulfur in the total solids.

Both protein and sulfur were higher in seeds from some plots than in those from others. This variation was highly significant when calculated either as content of moist beans or as content of bean solids.

The plants from one plot whose seeds were high in protein yielded consistently larger-than-average beans with relatively heavy pod hulls.

A highly significant direct correlation coefficient was found for protein and sulfur contents of lima beans.

An inverse but highly significant ratio was shown to exist between protein and water, and also between sulfur and water, in lima beans.

Water blanching and color sorting lowered only slightly the protein and sulfur per cent in the total solids.

CONCLUSIONS

Environment and water balance, rather than heredity, determine the levels at which nitrogen and sulfur occur in immature, green lima beans.

There is slight change in the nitrogen and sulfur fractions of the total solids because of removal of white, immature beans or because of blanching, freezing, and drying processes.

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EFFECTS OF WAXING ON CERTAIN PHYSIOLOGICAL PROCESSES OF CUCUMBERS UNDER DIFFERENT STORAGE CONDITIONS[1]

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Treatment of certain fruits and vegetables with wax emulsions now is an established practice in the commercial distribution of these commodities. According to Smock (1939), from 80 to 90 per cent of the citrus fruits shipped from California, by estimate, and almost as great a proportion of Florida citrus shipments are wax treated. Among commercial vegetables root crops often are waxed for market distribution. Rutabagas are dipped in melted paraffin, Harvey (1939); carrots are treated with wax emulsion, Platenius and Morris (1939); tomatoes, Anonymous (1937), and cucumbers, Platenius (1939) and Platenius and Morris (1939), are dipped in dilute wax emulsions.

The effects have been studied of wax treatment on certain physiological processes, chiefly those associated with deterioration in market or edible quality. A reduction in the shrinkage caused by transpiration, assumed to be the greatest cause of weight loss, has been observed by Hardenburg and Platenius on potatoes (1939); by Hitz and Haut (1939) on apples; by Jones and Richey (1939) on tomato plants and apples; by Nelson (1939) on apples; by Platenius (1939) on several kinds of vegetables; by Smock (1939) on apples, peaches, and nectarines; by Wardlaw and Leonard (1937) on grapefruit; and by an anonymous writer (1937a) on tomato fruits. All of these investigators observed considerable reductions in weight loss in storage of the respective waxed products, in comparison with untreated controls; where the reductions were reported quantitatively, they ranged from 20 to above 50 per cent of the loss by the unwaxed controls. In beets, however, decrease in weight loss was not so marked—Platenius (1939).

The influence of waxing on respiration was studied in fewer instances than was weight loss. Smock (1939) stated that treatment of apples and pears with wax emulsion reduced respiration; Brooks (1938) observed an increase in the CO_2 of the internal atmosphere of tomato fruits as a result of coating the stem scar with wax emulsion; Platenius and Morris (1939), however, found no effect of the treatment on the gaseous exchange through the epidermis of waxed vegetables in comparison with unwaxed controls.

Physiological breakdown and fungous spoilage also have been studied as well as ripening, in connection with wax treatment. Platenius (1939)

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stated that wax treatment reduced the extent of pitting of cucumbers in cold storage, a characteristic low-temperature injury of this and certain other fruits and vegetables described or observed by several investigators —Jones and Kubota (1940); Kidd and West (1933); Morris and Platenius (1939); Nelson (1926); and Rose, Wright, and Whiteman (1938). This reduction was attributed by Platenius to the maintenance of a higher moisture content in the tissues of the treated fruits. Fungous spoilage was reported by Iverson (1937) to be reduced in tomatoes by ozokerite wax treatment, except when the wax coating was broken; Wardlaw and Leonard (1937) also observed a reduction in fungous spoilage by wax treatment of grapefruit. Nelson (1939), on the other hand, stated that wax treatment of tomatoes encouraged rots, and Hitz and Haut (1939) found that scald was increased in apples by waxing immediately after harvest.

Effects of wax treatment on other processes of fruits and vegetables include also a relative delay in ripening and senescence, observed by Brooks (1938) and an anonymous author (1937a) on tomatoes and by Smock (1936) on apples and pears; development of alcoholic off-flavors, reported by Smock (1936, 1939) in apples and pears as a result of anaerobic respiration, although Brooks (1938) found no effect on flavor of tomatoes and Nelson (1939) stated that wax treatment improved the flavor of Patten Greening and Wealthy apples during the first day after removal from cold storage through a reduction in the musty flavor acquired by untreated apples; and improvement in sprouting and subsequent yield of seed potatoes, found by Hardenburg and Platenius (1939).

In the experimental studies reported herewith, which were carried out during the summer of 1939, observations were made on weight loss, sugar loss, carbon dioxide production, respiratory ratio, and spoilage from different causes in waxed and unwaxed cucumbers under three types of storage—in an ordinary room, in a bank cellar, and in refrigerated storage.

EXPERIMENTAL PROCEDURE

Greenhouse cucumbers of grade No. 1 Small were used. These were about seven inches long and two inches in diameter, free from injury, harvested during the early forenoon, and stored in ordinary unlined, square-woven, veneer market baskets with lids in cellar storage overnight before being placed under the experimental conditions. The variety was one bred and maintained by the growers, C. W. Zuck and Sons, of Erie, Pennsylvania.

Waxed ones were treated with a water emulsion of a wax mixture containing 75 per cent of carnauba and 25 per cent of paraffin, designated Brytene No. 284-D by the manufacturer, the Franklin Research Company, of Philadelphia, Pennsylvania, to which thanks are due for supplying the wax emulsion for these studies. The directions of the manufacturer were followed in the wax treatment; the emulsion, stated by the manufacturer to contain 35 per cent of the wax mixture, was diluted at the rate of one part by volume of the emulsion as received to six of tap water, and cucumbers were immersed until thoroughly covered, then removed and allowed to drain and dry.

Fifty-six cucumbers were placed under each storage condition; of these, half were waxed and the other half were not. Waxed and control lots were kept in separate baskets. Each fruit was numbered and all measurements were made on the basis of individual fruits.

Initial weights were recorded for all fruits, and weight losses were determined on all fruits remaining at the end of each interval of observation. Initial sugar content was determined separately for each of six cucumbers taken at random from the entire lot. Subsequent sugar analyses were made separately on each of three fruits taken from each of the waxed and control lots under each storage condition.

Respiration measurements were made individually on five cucumbers taken to represent each lot of fruit, from which no samples were removed for sugar analyses. The same fruits were used for respiration studies throughout the investigation, unless they became seriously damaged, in which event the remaining sound ones only were used. Initial respiration measurements were made at the end of the first day in the room and the bank cellar and during the second day in the cold storage. Respiration measurements were made under the conditions of storage for each lot except for those in cold storage. For the measurements in cold storage the respiration chamber (described later) was immersed in water maintained at the same temperature as that of the cold storage, to permit the observer to make the measurements in a more comfortable location.

Storage Conditions: The temperature of the ordinary room ranged from 21.1 to 29.4°C.(70 to 85°F.), and averaged 75°F.; the relative humidity thereof ranged from 58 to 67 per cent and averaged 63 per cent. In the bank cellar the ranges of temperature and relative humidity, respectively, were from 12.8 to 20°C.(55 to 68°F.) and from 80 to 90 per cent. Cold storage temperatures ranged from 2.2 to 3.3°C.(36 to 38°F.) and relative humidities between 95 and 99 per cent. The ordinary room and the cellar storage were dimly illuminated with diffused daylight during the day, and the cold storage was dark at all times except while fruits were being weighed or handled. Temperatures at the time when respiration measurements were made varied considerably less than the range stated above; an effort was made to take measurements within a range of five to seven °F. The temperature range during any one series of measurements on the treated and untreated cucumbers, under a single storage condition, was not over two or three °F., so that comparisons between the two treatments in any one week were not subject to wide temperature differences.

Weight Loss: The weekly weight loss of each fruit was recorded in percentage of weight of the respective fruits at the beginning of the experiment. Weights were measured to the nearest .01 gram.

Sugar Content: Analyses for content of total and of reducing sugars were made weekly on each lot, represented by three fruits taken at random. Each fruit was analyzed separately, by the picric acid-reduction method, as modified by Willaman and Davison (1924) from that originally suggested by Thomas and Dutcher (1924).

Respiration Rate: The rate of respiration was estimated weekly on each of five fruits representing each lot, by means of an absorption and titrating apparatus similar to that used by Mack (1930). As stated previously, the same fruits were used throughout the experiment unless they

were damaged by any cause to such an extent as to render them unsalable in the ordinary market.

The respiration chamber used was a 14-inch section of 2¾-inch steel pipe closed at each end with a rubber stopper through which a glass tube was passed to serve as an inlet or outlet for gases. Air freed from carbon dioxide was passed continuously through the respiration chamber at the rate of about 20 liters an hour, and respiration rate was determined from the carbon dioxide produced during a period of one-half hour.

Respiration Quotient: For measuring the respiration quotient, CO_2/O_2, a special respiration chamber was employed. This consisted of a section of steel pipe like that used for determining respiration rate but lined with a rubber sac of approximately the same outer diameter as the inner diameter of the steel pipe and about two inches longer than the steel pipe. This sac was open at one end, which was turned back in the form of a sleeve over the end of the steel pipe which it fitted rather tightly. Both rubber sac and steel pipe could be closed simultaneously with an air-tight connection by forcing a rubber stopper tightly into the open end of the sac within the end of the steel pipe.

The other end of the rubber sac was sealed tightly to a glass tube, providing an inlet into the sac through a rubber stopper closing the corresponding end of the steel pipe. A second glass tube through this stopper provided a vent between the outer air and the space surrounding the rubber sac within the steel pipe. When air was exhausted from this space through the vent, air would fill the rubber sac through the glass tube connected to it, at the prevailing atmospheric pressure; when air was forced into the space surrounding it, the air within the rubber sac was expelled. The rubber sac filled the space within the steel tube almost completely when air was exhausted from the tube; under this condition, diffusion of carbon dioxide through the rubber sac could be disregarded.

When the respiratory quotient was to be measured, a cucumber was inserted into the rubber sac and the open end thereof was closed as described above. A few quick changes of air were made in the rubber sac by alternate exhaustion and compression of the air in the space surrounding the sac, within the steel pipe, and the sac then was filled with air and closed. The cucumber was kept enclosed for 45 minutes, and the atmosphere surrounding it then was expelled as completely as possible into an Orsat gas-analysis apparatus graduated to .5 c.c., but capable of being read to .1 c.c. The respiratory quotient was taken as the ratio of the increase in CO_2 content to the decrease in O_2 content of the air from the rubber sac, as compared with that of the air of the room in which the measurements were made.

DISCUSSION OF RESULTS

Weight Loss: The mean weight losses, in percentage of the original weights of the respective fruits under the different conditions, during the successive intervals of approximately a week each and during the entire period are presented (Table 1). In each interval, the mean weight loss in per cent is the average of the percentage weight losses of the individual fruits which survived until the end of the respective intervals.

It is evident that the mean percentage weight loss of the waxed lots of cucumbers was less under each storage condition and during each interval than was that of the respective unwaxed lot, and in most cases the difference is significant. The greatest proportional reduction in weight loss by wax treatment in comparison with no waxing was in the ordinary room, in which it was approximately one-third. The least reduction by wax treatment was in the bank cellar.

Weight loss of unwaxed cucumbers in the ordinary room was more than double that in the bank cellar and in the cold storage, between which there was little difference. Weight loss in the ordinary room was considerably greater during the first week than during later intervals; in the other storages that of unwaxed fruits increased in later intervals but that of the waxed fruits showed no consistent trend. It was observed on individual fruits that weight loss became relatively greater just before breakdown occurred.

TABLE 1

Mean Percentage Weight Losses of Waxed and Unwaxed Cucumber Fruits During Successive Weekly Intervals Under Different Storage Conditions

Interval	Ordinary room, 21.1-29.4°C.(70-85°F.)		Bank cellar, 12.8-20°C.(55-68°F.)		Cold storage, 2.2-3.3°C.(36-38°F.)	
	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed
	pct.[1]	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
1	4.02±.265	6.22± .731	1.76±.168	1.95±.103	1.41±.113	1.90±.155
2	3.00±.129	4.60± .259	1.61±.131	2.09±.179	1.81±.139	2.12±.162
3	2.43±.185	3.99± .400	1.93±.211	2.23±.127	1.56±.354	2.35±.312
Whole period	9.34±.571	13.61±1.042	4.70±.333	5.85±.359	4.66±.582	6.31±.699

[1] The value after the ± sign is the standard error of the respective mean percentage.

Dry Matter and Sugar Content: The mean percentages of dry matter and of total and reducing sugars at the end of the different intervals indicated, under the three storage conditions, are presented (Table 2). The mean values presented are averages of the respective percentages of three fruits taken at random from each lot.

Relatively high percentages of dry matter were maintained in unwaxed fruits under all storage conditions, except in the bank cellar during the last week; in waxed fruits, on the other hand, dry matter was lower than at the beginning of the experiment in the ordinary room and bank cellar, except in the room at the end of the first 10 days. The dry matter of waxed fruits in cold storage, however, throughout the storage period remained either equal to or higher than that at the beginning.

That some of the differences in mean percentage of dry matter may be the result of sampling is indicated by the standard error; in general, a difference of about 12 to 15 per cent of the mean percentage would be required for significance.

The percentages of sugar in the fresh weight of the cucumbers show no consistent relationship to wax treatment. In the ordinary room and the bank cellar the percentages of sugars diminish throughout the period of the experiment to less than one-third of the original content in the ordinary room, at the end of the period, and to only a little over one-third

TABLE 2

Mean Percentages of Dry Matter, Total Sugars, and Reducing Sugars in Waxed and Unwaxed Cucumbers at Beginning of Experiment and at End of 10, 17, and 24 Days

Interval	Storage	Mean percentage of dry matter [1]		Mean percentage of sugars in fresh weight				Mean percentage of sugars in dry weight			
				Total sugars		Reducing sugars		Total sugars		Reducing sugars	
		Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed
days											
Initial	.....	.....	3.46	.....	1.639	.....	1.589	.....	47.36	.....	45.86
10	Room	3.65	3.46	1.350	1.075	1.271	1.003	36.98	31.08	34.82	29.00
	Bank cellar	3.13	3.49	1.193	1.365	1.176	1.364	38.11	39.12	37.56	39.10
	Cold storage	3.44	3.65	1.654	1.729	1.557	1.649	47.94	47.23	45.12	45.04
17	Room	3.14	3.71	0.973	0.945	0.937	0.913	30.98	25.39	29.85	24.53
	Bank cellar	3.13	3.33	1.125	1.055	1.031	0.991	35.94	31.68	32.95	29.76
	Cold storage	3.69	3.58	1.737	1.676	1.665	1.549	47.01	46.82	45.00	43.27
24	Room	3.10	3.36	0.516	0.523	0.429	0.467	16.63	15.57	13.83	13.89
	Bank cellar	2.91	3.10	0.947	0.677	0.817	0.562	32.53	21.84	28.08	18.13
	Cold storage	3.51	3.27	1.705	1.477	1.641	1.335	48.57	45.18	44.76	40.81

[1] Standard errors of the mean percentages of dry matter and of the mean percentages of sugar in the dry weight were computed; the former ranged from less than one per cent to eight per cent of the respective means, and of the latter from five to 20 per cent of the respective means. The highest standard errors were in the unwaxed cucumbers during the last interval, in which considerable spoilage occurred.

in the unwaxed fruits in the bank cellar. In the cold storage, however, the changes in sugar content are not consistent.

On the basis of dry weight, however, wax treatment apparently reduced sugar loss during all intervals in the ordinary room and cold storage, and during all except the first 10-day interval in the bank cellar.

Respiration Rate: The mean rate of carbon dioxide production in milligrams per hour per kilogram of fresh weight of the lots of five

TABLE 3

Respiration Rate in Milligrams of CO_2 per Hour per Kilogram of Fresh Weight of Waxed and Unwaxed Cucumbers Under Different Storage Conditions

Weekly interval	Ordinary room, 21.1-29.4°C. (70-85°F.)		Bank cellar, 12.8-20°C. (55-68°F.)		Cold storage, 2.2-3.3°C. (36-38°F.)	
	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed
Initial	21.32±2.03	23.80±1.37	20.49±1.72	19.74±1.22	5.69±0.56	8.28±0.30
1	26.71±5.03	23.87±7.16	16.93±0.82	16.29±0.77	13.09±1.69	19.58±1.57
2	15.40±2.05	12.11±3.04	16.61±4.29	13.42±1.62	15.40±3.93	8.34±1.25
3	12.22±0.42	9.91±1.23	9.79±0.79	6.88±1.22	20.34±0.00	22.11±5.39

cucumbers under each of the different experimental conditions is shown (Table 3). While respiration rate diminished rapidly after the first week in the ordinary room and after the second week in the bank cellar, it increased rapidly in cold storage, except in the second week in the unwaxed fruits. The diminishing rate of respiration in the ordinary room and bank cellar is associated with reduction in sugar content; the accelerated rate in the cold storage was associated with a high sugar content and with physiological breakdown. Individual fruits with incipient breakdown

TABLE 4

Mean Respiration Quotients (CO_2O_2) of Waxed and Unwaxed Cucumbers Held Under Different Storage Conditions

Weekly interval	Ordinary room, 21.1-29.4°C. (70-85°F.)		Bank cellar, 12.8-20°C. (55-68°F.)		Cold storage, 2.2-3.3°C. (36-38°F.)	
	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed
Initial	0.88±.01	0.82±.06	1.04±.01	0.73±.05	0.43±.04	0.47±.02
1	1.13±.41	1.10±.03	1.10±.11	0.97±.04	1.23±.04	0.86±.05
2	0.92±.05	1.10±.03	1.20±.17	1.14±.14	1.67±.30	1.01±.02
3	1.28±.37	1.53±.17	1.07±.00	1.08±.24	1.22±.06	0.96±.05

were observed to have specially high respiration rates. Wax treatment apparently exerted no consistent, significant influence on respiration rate.

Respiration Quotient: The mean respiration quotients, CO_2/O_2, of the lots of five cucumber fruits under each of the experimental conditions are presented (Table 4). Initial quotients less than unity were observed in all lots except the waxed ones in the bank cellar; the initial quotients in cold storage were very low. With several exceptions the values of the quotients increase in later intervals and are specially high in the ordinary room at the end of the experimental period and in the waxed fruits in cold storage, after the initial measurement. The only significant differ-

ence associated with the wax treatment is that in the cold storage; the respiration quotient is significantly greater in the waxed fruits than in the unwaxed ones, from the end of the first week until the end of the experiment.

Other Observations: Shrivelling was the first symptom of deterioration observed in the fruits stored in the ordinary room; this appeared at the end of the first seven days on unwaxed fruits. During the second week fungous rots destroyed a considerable number of the unwaxed fruits in the ordinary room, and others though remaining in edible condition, developed numerous small, orange-colored, slightly raised spots somewhat resembling bacterial scab. Few of the waxed fruits in the ordinary room were discarded because of either shrivelling or fungous rots, though the orange-colored spots appeared during the second week as they did on the controls. Both waxed and unwaxed fruits began to turn yellow about equally at the end of three weeks in the ordinary room, at which time 14 waxed fruits and six unwaxed ones remained of a possible 19 in each lot.

In the bank cellar no difference was observable between the waxed and the unwaxed lots, either in the incidence and extent of shrivelling or of the orange spots described above; the former occurred at the end of the first week, and the latter appeared during the second week. Fungous rots developed earlier in the waxed fruits than in the unwaxed ones, and yellowing began at about the same time in both lots or after two weeks. At the end of three weeks, 14 waxed and 12 unwaxed fruits remained.

In cold storage, pitting appeared during the first week but was more severe on waxed fruits than on unwaxed ones, as were also fungous rots, which developed during the second week. Neither shrivelling, yellowing, nor the orange spots mentioned above appeared on either lot in cold storage. At the end of the three weeks only four waxed and eight unwaxed fruits remained of the original 19 not used for sugar analyses.

DISCUSSION

The reduction in weight loss effected by waxing evidently was due to decrease in transpiration, in view of the fact that respiration rate was not significantly affected by the wax treatment. Further evidence is furnished by the lower percentage of dry matter, i.e., greater percentage of moisture, in the waxed fruits in the ordinary room and in the bank cellar, in which the greatest reductions in weight loss were brought about by wax treatment.

Decreases in dry-matter content of waxed fruits in the ordinary room and the bank cellar could occur only if the proportional loss of dry matter by respiration or volatilization were greater than that of water by transpiration. That this is not an impossibility is indicated by the fact that, at the rate of approximately 20 mg. of CO_2 per hour, 3.36 gm. of CO_2 per kg. of fresh weight would be lost in a week, or approximately 10 per cent of the dry matter present. Because percentage weight losses were always less than this, it is apparent that the percentage of dry matter would be subject to reduction.

The relatively high respiratory quotients of waxed fruits in cold storage indicate a relatively low oxygen consumption by the fruits under this

condition. It is to be expected, therefore, that pitting would be more severe in these fruits, if the disease is related to suboxidation.

SUMMARY

In the foregoing report the effects are described of treatment with a wax emulsion on the weight loss, dry-matter content, sugar content, respiration rate, respiratory quotient, physiological breakdown, and changes in physical appearance of greenhouse cucumbers stored during three weeks in an ordinary room in midsummer, in a bank cellar, and in cold storage. The most striking differences brought about by wax treatment were reduction in weight loss and in spoilage in the ordinary room; increase in pitting in cold storage, a characteristic low-temperature injury on cucumbers; and a significant increase in the respiratory quotient in cold storage, indicating a greater degree of suboxidation.

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NATURE OF DARKENING OF COOKED POTATOES

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The darkening of potatoes after cooking has been a source of concern for many years. It is one of the most common complaints made by consumers, according to a recent survey conducted by Hotchkiss, Wood, and Findlen (1940). In an effort to further understanding of the cause of this difficulty, a study has been made of the conditions under which cooked potatoes discolor and the factors involved in the blackening process.

The terms *darkening* and *blackening* are in general use with reference to several discolorations that may occur in cooked potatoes. One type of discoloration is caused by the action of pathogens. Another type, apparently caused by substances present in the skin, appears as a brownish-yellow color on the outer surfaces of cooked tubers after the skins are removed and is absent when tubers of the same variety are pared before they are cooked. In the present work these two types of discoloration are not considered further. Two additional and distinct types of darkening of cooked potatoes, however, occur; the difference between them is clearly marked (Fig. 1).

One type of darkening is observed under several different conditions. Some varieties of potatoes, when cooked, show dark spots around the eyes (Fig. 1a) even though the tubers have been pared before cooking; in others the tissue around digger cuts or other injured portions frequently becomes dark. Sometimes both occur on the same potato (Fig. 1b). In both cases small particles of a brown or brownish-black substance are observed on the surface of the cooked potato. When magnified the particle nature of such darkening is very evident (Fig. 2). The dark particles become apparent early in the cooking process; the discoloration does not increase in intensity after the tuber is allowed to cool. Dark particles similar to those illustrated may also appear during cooking if, before cooking, a pared potato is exposed to air until a red color appears on the surface. Such darkening is often seen on potatoes which have been pared before cooking at the place where the tuber was attached to the stolon. Apparently the dark-particle formation had begun before the tubers were cooked. If oxygen is necessary to the final color change, the amount is not greater than the potato itself can supply since the change occurs even while the tubers are in rapidly boiling water.

The dark particles are stable and are not dissolved by solvents, such as alcohol, ether, or dilute acid. Dilute alkali produces general discoloration and disintegration of the tuber. Warm alkaline hydrogen peroxide destroys the color of the particles. The brownish-black darkening may be due to the formation of a melanin. Such a possibility is suggested by the work of Raper (1928) and his coworkers; they produced melanin using the tyrosinase of raw-potato juice and tyrosine, and postulated

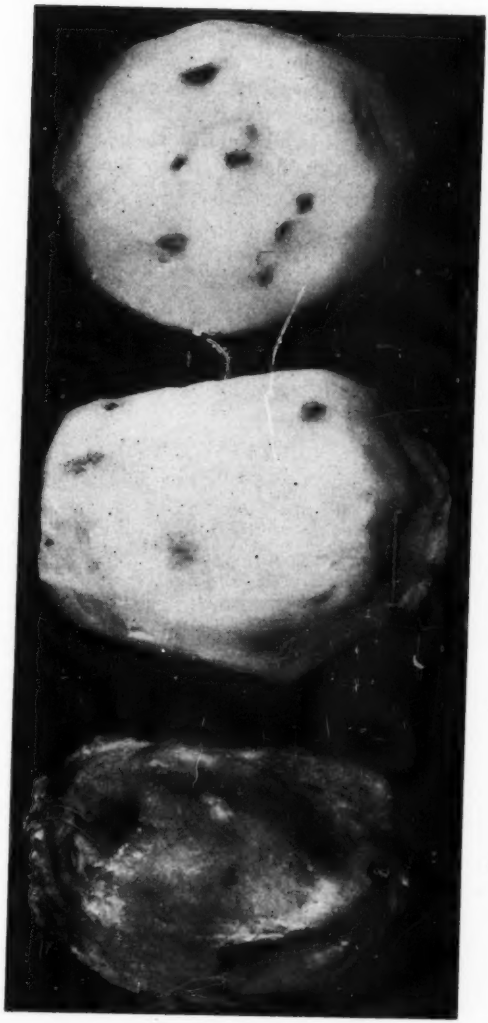


FIG. 1. Two types of discoloration on potatoes. Top to bottom: a. particle eye-darkening (Warba); b. particle eye-darkening and bruises (Green Mountain); c. stem end view of diffuse blackening (Green Mountain).

that a series of enzymatic and non-enzymatic oxidations and molecular rearrangements were involved. The properties of the dark particles are, moreover, similar to those of melanin, described by Jacobsen (1934). In addition, the observations made on cooked tubers are in accord with the work of Scott (1936), who found that the formation of a melanin might be brought about by such agencies as age, frost, and bruising, which kill part of the tuber but do not inactivate the enzymes. Szent-Györgyi and Vietorisz (1931) showed that the activity of the phenoloxidase of potatoes was increased after mechanical injury of the tissue. In general, when the darkening due to the brownish-black deposits occurs, it covers only a small area on the surface of the potato and can be cut away without appreciable loss.

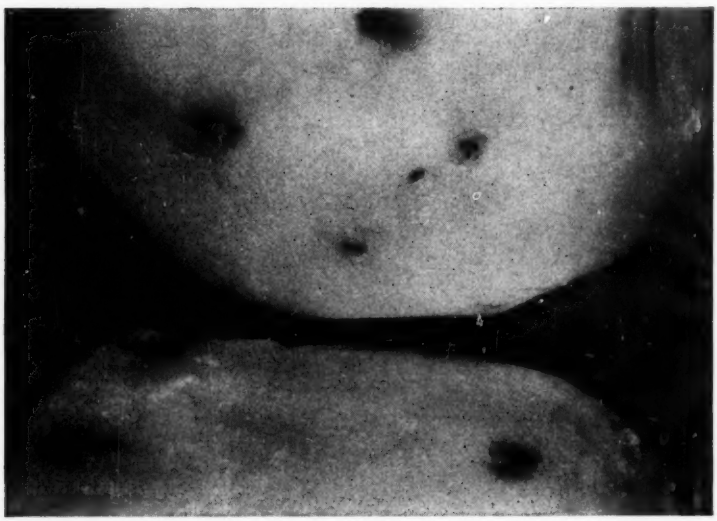
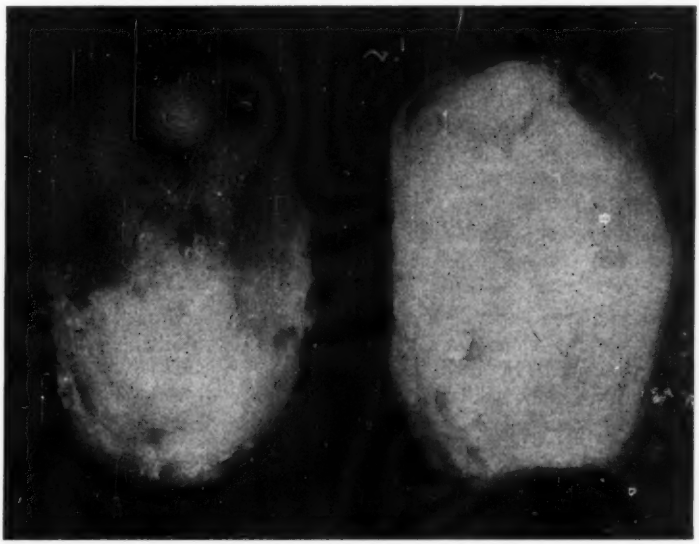


FIG. 2. Enlargement of portions of Fig. 1a and b showing brownish-black particle formation (×2).

The second type of darkening, however, results in the formation of a diffuse bluish-black color usually centering in the stem end of the cooked tuber (Fig. 3). Such blackening may so extend on the surface and within the tuber that in extreme cases as much as one-third of the potato is rendered unattractive and unappetizing. The blue-black stem-darkening is the primary consideration of this work and will be designated as *blackening* as has been done by some other workers.

There is apparent disagreement among workers with regard to the process involved in stem-blackening. Tinkler (1931) made the statement that it was extremely probable that the blackening process was an oxidation, since some tubers, which ordinarily blacken after cooking, do not discolor when placed in gas, free from oxygen, immediately after cooking.

Both Ashby (1905) and Tinkler (1931) expressed the opinion that blackening after cooking could not be due to the formation of melanin. On the other hand, several recent workers in the field—notably, Hansen (1936), Tottingham, Nagy, and Ross (1936, 1937, 1938, 1939), Smith and Nash (1938), and Nash (1940)—apparently assume that the formation of a melanin is involved, although at least some of these workers accept the fact that the final steps in the process cannot be enzymatic. The present work was undertaken with a view toward acquiring evidence that might clarify the situation.



a. b.

FIG. 3. a. Potato (Green Mountain) showing stem-blackening. b. Same variety showing no blackening.

EXPERIMENTAL PROCEDURE

In order to study the blackening of cooked potatoes and the supposed oxidation process in detail, potatoes were boiled or baked and then cooled in an atmosphere of nitrogen; potatoes were also cooked in water to which various chemicals had been added. The potatoes selected were from groups that had previously shown a tendency to blacken.

For the cooking of potatoes in water, equal portions of tap water (350 ml.) were boiled for about 10 minutes in each of two beakers covered with two-holed glass covers. By means of a glass tube, which reached to the bottom, nitrogen was introduced into one beaker during the last five of the 10 minutes and was bubbled steadily and continuously through the water until the end of the cooking period. One half of a single, thinly pared potato cut lengthwise was placed in each beaker and cooked until tender. Then, while a rapid stream of nitrogen was forced into the

beaker, as much of the water as possible was siphoned from the one potato half and the remainder of the water was poured off without removal of the cover. All of the water was poured from the other potato half and this beaker also was left covered. The beakers were cooled for 30 minutes at room temperature and subsequently for an additional 15 minutes surrounded by ice water. The halves, one still in an atmosphere of nitrogen, were examined for blackening while they remained in the beakers. Finally, they were removed to the air and again compared after they had stood at room temperature for approximately one hour. Thirteen tubers representative of five different varieties were cooked.

The baking process was carried out in an atmosphere of nitrogen in a small improvised tin oven through which a stream of nitrogen was passed. Single, unpared potatoes were baked at approximately 210°C.(410°F.), partially cooled in the nitrogen-filled oven and then removed as quickly as possible to a gas-tight box filled with nitrogen. The box was equipped with a glass window and two rubber gloves so attached that the potato could be manipulated in the gaseous atmosphere. The baked potato was cut in half, carefully pared, and allowed to cool to room temperature. The cold tuber was examined for blackening while in the box and again after exposure to air. Seven tubers representative of three different varieties were used.

Halves of pared tubers were cooked in rapidly boiling water (450 ml.) to which various chemicals had been added, while the corresponding halves were cooked in a similar quantity of tap water. After cooking, the tubers were removed from the water to the air and, as soon as they were cold, the two halves were compared for blackening.

DATA AND DISCUSSION

Although none of the 13 potato halves that were cooked in untreated water showed blackening during cooking, all but one of the halves blackened during cooling in air; four showed slight, six intense, and two very intense blackening. The intensity of the blackening increased to a maximum as the potatoes stood in air and gradually cooled to room temperature. There was no further increase after the tubers were taken from the beakers and allowed to stand, even if they were placed in the refrigerator. Such behavior is in accord with the general observations made in this laboratory on many pared, steamed, as well as boiled, potatoes. In each case the blackening attained its maximum as the potato remained in contact with the air. The maximum was reached in less than an hour and showed no apparent increase either on further standing or on reheating by steaming as was done in some cases; on the contrary, the blue-black darkening sometimes faded. If, for example, a freshly cooked potato showing stem-blackening was placed in a container which was tightly covered so that moisture condensed on the tuber, the blackening frequently decreased. Any eye-darkening on that same tuber, however, was stable and remained dark. Occasionally brownish-black particles and diffuse blackening were superimposed on one another in the same area of the potato.

In contrast to the behavior of the potato halves cooked in untreated water none of the corresponding halves cooked in oxygen-free water and then cooled in an oxygen-free atmosphere showed blackening. When these potatoes were later exposed to air, however, they all showed a definite blackening that in all but two cases became as intense as that on the potato half cooked in untreated water and cooled in air. The blackening appeared as soon as the halves were exposed to air and attained a maximum over a period of an hour or less. The only color change of any kind observed was the appearance and intensification of the blue-black darkening. The seven tubers that were baked, cooled, and pared in an inert atmosphere showed no appreciable blackening as long as air was excluded. As soon as the tubers were removed to the air, however, all but one blackened, two slightly and four intensely.

The stem-blackening of potatoes can thus be definitely attributed to oxidation but, in view of the fact that the internal temperature of the cooked potato is 97.8°C.(208°F.), the probability of an enzymatic oxidation is slight. In any case the oxidation cannot involve the enzyme tyrosinase since, according to Gallagher (1923), the activity of this enzyme is destroyed after 10 minutes' heating at 75°C.(167°F.). The oxidation may involve a substance already present in the raw tuber which can act or be acted upon after the structure of the potato is loosened during cooking. It is equally possible that during cooking a substance is formed which itself can be oxidized or which in the presence of oxygen can oxidize a component of the potato. Support for these suppositions is indicated by the fact that mealy potatoes, which tend to slough more than do soggy ones during cooking, have been observed in this laboratory to blacken frequently, whereas in general soggy potatoes do not blacken.

Several of the potato samples showed brownish-black eye-darkening when cooked in the boiling water through which nitrogen was bubbled. At times eye-darkening was preceded by the formation of a reddish-brown coloration on the raw tuber. The darkening appeared during the boiling process and was not noticeably intensified after the tubers were removed to the air. Apparently the formation of the brownish-black pigment had begun even before the potato was cooked. This behavior is in agreement with the general observations already given for the formation of melanin. It must be assumed, however, that in these cases the quantity of oxygen necessary for the final oxidation is supplied by the tuber while it is in the boiling water.

A series of chemical agents, including oxidizing and reducing substances, metals, sugars, amino acids, other organic acids, and mineral acids, were used in the water in which pared potatoes were cooked without significant results except in the case of the acids. When the solution was acidic the tendency to blacken was markedly decreased or even overcome (Table 1). A quantity of acid sufficient to give a pH value of the water before cooking of 4.1 or less and after cooking of not more than 4.9 resulted in an appreciable decrease in the intensity and extent of the blackening. Unfortunately at the same time a thin, tough layer was formed on the outside of the potato. Even though the tuber might not taste noticeably sour it was rendered quite unpalatable by the tough layer.

When the quantity of acid used was increased, blackening was decreased but the thickness of the objectionable layer was more pronounced. Moreover, in cases of extreme blackening discoloration was usually found underneath the tough layer. Potatoes that appeared light yellow when cooked in boiling water were usually creamy white when cooked in acid.

These observations in regard to the effect of acid are similar to those made by Gleason and Marlatt (1934) and Hansen (1936), who state that acid decreases the discoloration and makes the potato less tender. No experimental data are given, however. The eye-darkening of the tubers cooked in acid solutions was also decreased. Thus in this particular respect, eye-darkening and stem-blackening behave in a similar fashion. Raper and Wormall (1923), while investigating the formation of melanin

TABLE 1

Effect of Acidified Cooking Water on Stem-Blackening of Boiled Potatoes[1]

Half in water	Half in acid	Number of cases		
		Citric acid[2]	Lemon juice[3]	Vinegar[4]
Slight blackening	No blackening	9	6	6
Intense blackening	No blackening	3	3	4
Intense blackening	Slight blackening	4	7	3
Very intense blackening	Slight blackening	1	2	3
Very intense blackening	Intense blackening	3	0	4
Very intense blackening	Very intense blackening	0	2	0

[1] Halves of smooth rurals, russet-rurals, heavyweight, and unknown varieties were boiled in acid solutions as compared with halves of same tubers boiled in untreated water. Sixty tubers from the crop of 1939 were tested in 1940. [2] 2 tb./qt. citric acid (.1 M); pH before cooking 3.7, after cooking 4.6-4.9. [3] 2 tb./qt. strained lemon juice; pH before cooking 3.3, after cooking 3.9-4.2. [4] 2 tb./qt. cider vinegar; pH before cooking 4.0-4.1, after cooking 4.6.

in vitro, noted that at pH values of less than five there is no appreciable reaction between tyrosine and tyrosinase.

In order to determine whether the similarity in behavior at low pH values might be fortuitous, some tubers were cooked in alkaline solutions, since Raper and Wormall (1923) also state that the formation of melanin is slight at a pH of nine and ceases when the pH value exceeds 10. When potato halves were boiled in water containing one-half teaspoon of baking soda to one quart of water, the pH values of the waters at the beginning of the cooking period were between 8.4 and 8.9 and after cooking between 9 and 10. The intensity and extent of the blackening of the potato halves cooked in the water containing soda was the same as or greater than that of the control halves. Of the 20 potatoes tested, seven halves showed the same degree of blackening when cooked in water containing soda as did the control halves cooked in tap water. The halves of the remaining 13, however, cooked in water containing soda, tended to blacken more than did the corresponding halves cooked in tap water; five showed more intense blackening, four showed more extensive blackening, and four showed both more intensive and extensive blackening. There was some browning on the exterior of certain of these tubers, presumably due to decomposition. When larger quantities of soda were used, the decomposition and surface browning became very marked but the intensity of the black-

ening in the interior was not decreased. In contrast to the increased development of stem-blackening, in the presence of soda, eye-darkening was never intensified and at times appeared less intense. The persistence and even increase of stem-blackening in highly alkaline solutions is unlike the behavior that would be expected if blackening were due to a tyrosine-tyrosinase reaction.

SUMMARY

Evidence has been presented to show that the blue-black diffuse stem discoloration of cooked potatoes, designated as *blackening*, is the result of an oxidation. The oxidation cannot involve the enzyme tyrosinase or any other enzyme the activity of which is destroyed at 97.8°C.(208°F.). Blackening is a process distinct from the one that results in the formation of brownish-black particles around the eyes of tubers or at injured surfaces. Diffuse blackening was found to be less stable than the brownish-black particles. Blackening was markedly decreased or prevented by the use of cooking waters of pH 4.1 to 4.9, but a tough layer was formed on the outside of the tuber. Blackening was unchanged or slightly increased by the use of cooking waters of pH 8.4 to 10, but in such alkaline solutions some surface decomposition and browning occurred.

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APPLICATION OF VOLATILE FATTY ACIDITY DETERMINATION TO A STUDY OF CANNED MAINE SARDINES

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Numerous methods of evaluating the relative freshness of fish have been proposed. The methods may be grouped into three general classes: (1) organoleptic methods in which the quality is judged on the basis of appearance, odor, and taste; (2) bacteriological methods in which the total count and types of bacteria are useful for the evaluation of freshness; and (3) chemical, physical, and physical-chemical methods in which the presence of certain breakdown products, changes in conductivity, elasticity, buffering capacity, and other factors are used as a measure of quality. For a thorough discussion and review of the literature of the various methods, Loeffler's thesis (1938) should be consulted.

Organoleptic methods when applied by experienced people are unquestionably of value since that is the basis on which the consumer is going to judge the product, regardless of what chemical or other tests may show. However, there are no organoleptic standards for comparison and the personal factor prevents consistently good agreement between individuals or even in the same individual on different occasions.

Total bacterial counts on fresh fish may be used as a criterion of decomposition but the time involved in making the counts and the fact that sometimes part of the decomposition is autolytic makes the method impractical under some conditions. Furthermore, in canned products almost all the microörganisms are killed by the heat treatment so that total counts would not be significant.

Of the chemical and physical-chemical tests which have been proposed the method of Stansby and Lemon (1933), used to measure changes in buffering capacity, and determination of volatile basic nitrogen compounds, particularly trimethylamine as suggested by Beatty and Gibbons (1936), have been used most successfully to gauge the quality of raw fish. Loeffler (1938) reports, however, that the methods used on fresh fish could not be applied satisfactorily to canned fishery products because of changes in the latter during processing and storage.

Hillig and Clark (1938) and Loeffler, working independently, found that volatile fatty acidity was the most useful index for the determination of quality in canned fishery products. The test was applied by Hillig and Clark (1938) to canned salmon, tuna, and mackerel; and by Hillig (1939a) to herring roe and (1939b) to California sardines; while Loeffler, using a slightly different technique, worked only with salmon.

The object of this investigation was to determine the effect of various factors, such as bacterial count, brine concentration, method of holding, and processing, on the development of volatile fatty acidity in canned

sardines. The method of Clark and Hillig was also compared with that of Loeffler.

EXPERIMENTAL PROCEDURE

The method of Clark and Hillig (1938) was used to obtain all data other than that used for a comparison of the two methods, because the preliminary experiments were made by that method and the results were found to be satisfactory. Since some of the work was done at a field laboratory where gas was not available, electric hot plates were substituted for gas burners to maintain a constant level in the distilling flasks. Temperature of the hot plates was controlled with a rheostat and the rate of steam flow was regulated with a Variac. It was possible to run two determinations at one time from a single steam generator of the type recommended by Clark and Hillig (1938).

Briefly, the procedure consists of steam distillation of an acidified filtered extract of the ground sardines. Phosphotungstic acid is used to precipitate the protein. The volatile acid number is the calculated number of cubic centimeters of .01 N. alkali required to neutralize the volatile acid in 200 c.c. of distillate from 100 grams of fish, although only a 50-gram sample is taken. The distillation is specified to be at the rate of 200 c.c. an hour with the liquid in the distilling flask kept at a constant volume.

The method is arbitrary and does not attempt to measure the total volatile fatty acidity of the sample. Hence it is important that all conditions should be kept as uniform as possible for all determinations. Specific directions may be found in the papers by Clark and Hillig (1938) and Hillig and Clark (1938).

RESULTS ON 1939 PACKS OF SARDINES

The results of volatile fatty acid determinations made on sardines packed in 1939 are shown (Fig. 1). The sardines were held under various conditions prior to canning and processing and the tests were made on the canned samples after a storage period of from seven to 10 months.

Sardines held in a strong brine before packing showed in general little increase in volatile acidity even after a holding period of as long as 50 hours. Curves 9 and 10 show this point clearly. The salt content of the pack represented by 10, 32° salometer, was about half that of 9, 64° salometer, after 50 hours, which is the probable explanation for the higher volatile acidity in the 10 sample. Curve 8 shows the result of holding the sardines in sea water treated with chloroform as compared with fish held in untreated sea water (Curve 7) or brine (9 and 10). The chloroform was added to inhibit bacterial growth and still permit the naturally occurring or autolytic enzymes present in the fish to continue their action. Judging from these results the strong brine not only prevented bacterial action but also decreased autolysis. The fish held in untreated sea water started developing volatile fatty acids after 20 hours' holding, which it was assumed was due to bacterial action plus autolysis. Sardines held in chloroform-treated sea water did not develop an appreciable quantity of volatile fatty acids until after 45 hours.

A comparison of Curves 2 and 3 shows the effect of holding sardines in the unsealed cans on the production of volatile fatty acidity. The

fish represented by Curve 2 were held on the flakes, while those in Curve 3 were held in the cans. At the end of 48 hours the fish held on the flakes had shown but little increase in the volatile acid number while the value of those held in the cans had more than doubled.

Curve 6 shows the same result more strikingly. The open cans of fish were held up to 48 hours in a warm room. Even after 24 hours the

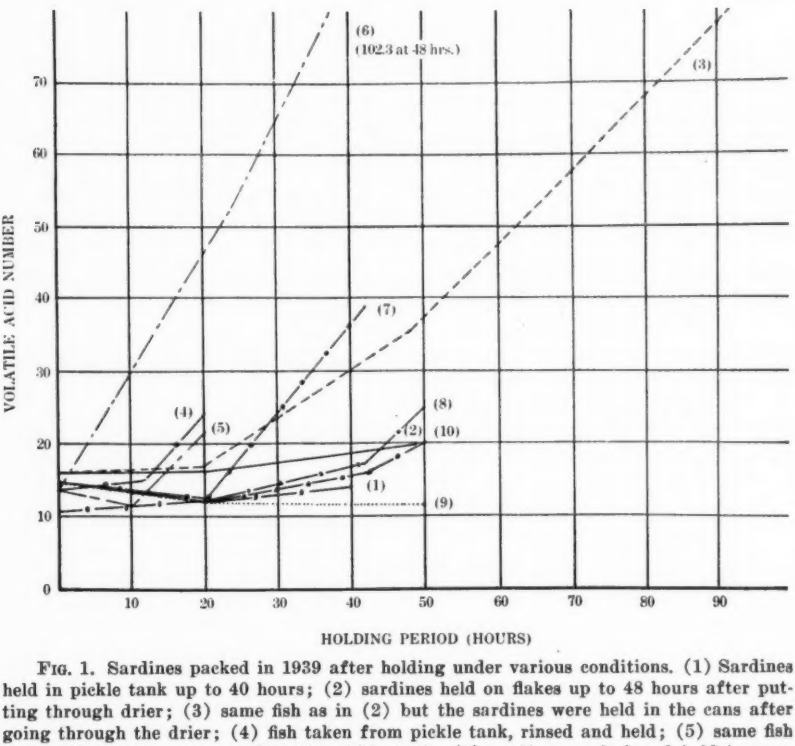

FIG. 1. Sardines packed in 1939 after holding under various conditions. (1) Sardines held in pickle tank up to 40 hours; (2) sardines held on flakes up to 48 hours after putting through drier; (3) same fish as in (2) but the sardines were held in the cans after going through the drier; (4) fish taken from pickle tank, rinsed and held; (5) same fish as in (4) but they were held in the pickle tank; (6) sardines packed and held in open cans without separators; (7) fish held in sea water; (8) same lot as (7) but held in sea water plus chloroform; (9) same lot as (7) but the fish were held in same brine used in hold of boat which tested 32° salometer; (10) same lot as (7) but fish were rinsed and held in pickle brine testing 64° salometer.

samples had developed an off-odor and the volatile fatty acid number was four times that of freshly canned sardines. The most plausible explanation for the more rapid increase in volatile acidity of fish held in the cans is that the sardines are relatively free from bacteria after steaming and drying but become contaminated during the handling at the packing tables. Volatile fatty acidity produced in such a way would probably be caused entirely by bacteria since the autolytic enzymes should be inactivated by the steaming process.

VOLATILE FATTY ACID DETERMINATIONS ON FRESH SARDINES

In the summer of 1940 the studies were extended to include determinations on fresh sardines. The necessary equipment was set up at the field laboratory in Eastport, Maine, where facilities were also available for making bacterial tests. For the volatile fatty acidity determination, four fish from each lot were headed and gutted. They were then ground through the food chopper four times and the sample for testing was weighed out.

Determinations were made on raw sardines and canned packs were made from the same lots after storage for one, two, three, and, in two cases, four days. It was not possible to make tests on all the lots at exactly the same time interval owing to limitations of equipment; for example, the samples tested after one day's holding period were actually from 19 to 29 hours old. After 24 hours the volatile fatty acidity was increasing rapidly in some lots. The time at which the determination was made was noted in most cases and allowance was made on the chart for this factor. Results of the determinations on the raw fish are shown (Fig. 2).

Curve 1. The sardines held in the ordinary sea water at a temperature of 15°C.(59°F.) showed some increase in volatile fatty acidity after 18 hours, but after 44 hours the fish were definitely decomposed and the volatile acid number had increased 15-fold.

Curve 2. These sardines were held in a 30° brine at a temperature of 15°C. By the time the brine reached an equilibrium the salt content was down to 20°. The development of volatile fatty acidity was delayed somewhat by this concentration of salt but for practical purposes the 30° brine was not much better than plain sea water.

Curve 3. When held in a 60° brine at 15°C. the volatile acidity number was only 46 after 72 hours as compared with a value of 106 for the sardines held in 30° brine or 145 for sardines held in untreated sea water. After equilibrium between the sardines and the brine had been reached the salt content was down to 30° which appeared to be approximately the lower limit for appreciably delaying volatile fatty acidity development.

Curves 4 and 5, representing the volatile acid number of fish held in 30 and 60° brine at a warm temperature, when compared with Curves 2 and 3 show how an increase in temperature affects development of volatile fatty acidity in sardines held in brine. At the end of 40 hours the volatile acid number of the fish held in the 30° brine at a temperature of 21°C. (70°F.) was 134, almost four times the value of 34 for fish held in the same brine at 15°C. The volatile acid number of fish held in the 60° brine at 21°C. was 44 at the end of 40 hours, while fish held in the same strength brine at 15°C. had a value of 24. The fish held in the 60° brine at the cool temperature had a much brighter appearance than any of the other lots.

Curves 6 and 7 show the effect of chloroform on volatile fatty acidity production at holding temperatures of 15 and 21°C. Chloroform definitely delayed the formation of volatile fatty acidity in sardines held both at the warm and cool temperatures. The volatile acid numbers for the

chloroform-treated samples were intermediate between the values for the 30 and 60° brine samples at both the high and low temperatures. Bacterial counts showed that the chloroform did not prevent bacterial growth (Table 4). Consequently on the basis of these results we are not prepared to say how much of the production of volatile fatty acidity is due to bacterial action and how much is due to autolysis.

Curve 8. The fish were held in sea water plus ice. The temperature ranged from 6 to 10°C.(43 to 50°F.). At the end of 48 hours the fish had a volatile acid number in the same range as the fish held in the cool

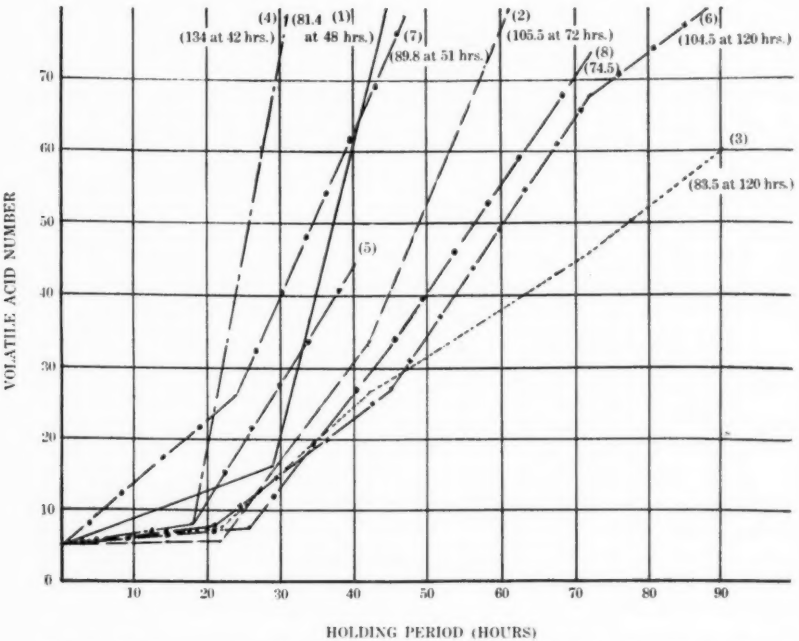

FIG. 2. Tests on raw sardines (1940). (1) Sardines held in sea water at 15°C. (59°F.); (2) sardines held in a 30°brine (20° after equilibrium) at 15°C.; (3) sardines held in 60° brine (30° after equilibrium) at 15°C.; (4) sardines held in 30° brine at 21°C.(70°F.); (5) sardines held in 60° brine at 21°C.; (6) sardines held in sea water plus chloroform at 15°C.; (7) sardines held in sea water plus chloroform at 21°C.; (8) sardines held in iced sea water.

30° brine and the fish held in chloroform-treated sea water. After 72 hours the fish held in ice had a volatile acid number of 75 compared with 68 for the fish in the chloroform-treated brine, 106 for the fish held in the 30° brine, and 46 for those in the 60° brine.

VOLATILE FATTY ACID DETERMINATIONS ON CANNED SARDINES

Canned packs were made from the lots described in the above section. These packs were tested for volatile fatty acidity within a month after

canning; results are shown (Fig. 3). As was noted previously, determinations on the fresh samples were of necessity spread over eight to 10 hours so a direct comparison with the canned samples is not too accurate because bacterial and enzymic action would be stopped in the latter when the fish was pressure-processed. In general, however, the canned samples packed after 24 hours had a slightly higher volatile fatty acidity than the corresponding fresh samples. After 72 hours' holding under various conditions, the majority of the canned samples had a lower volatile

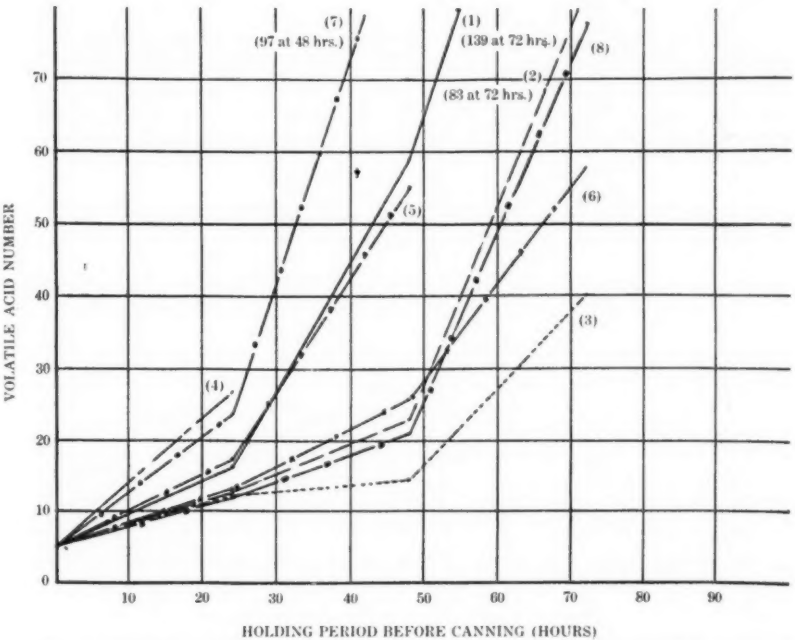

FIG. 3. Tests on canned sardines made up from same lots as represented in Fig. 2 (1940). (1) Sardines held in sea water at 15°C.(59°F.); (2) sardines held in a 30° brine (20° after equilibrium) at 15°C.; (3) sardines held in 60° brine (30° after equilibrium) at 15°C.; (4) sardines held in 30° brine at 21°C. (70°F.); (5) sardines held in 60°brine at 21°C.; (6) sardines held in sea water plus chloroform at 15°C.; (7) sardines held in sea water plus chloroform at 21°C.; (8) sardines held in iced sea water.

acid number than the corresponding raw sardines. A possible explanation for the lower volatile acid numbers in the older samples is that the acids formed were more volatile and there was some loss in the steaming process.

EFFECT OF PROCESSING ON ACID CONTENT

Volatile fatty acidity determinations were made on sardines taken from the brine tank, on a sample of the same lot of sardines after they had been packed in the cans and sealed, on a sample immediately after processing,

and after the sardines had been stored in the cans for two weeks. The results were as follows:

	Fish from brine tank	Fish after sealing but before processing	Fish after processing	Fish after two weeks' storage
Volatile acidity	6.7	8.2	11.7	10.7

There is apparently a small increase in volatile fatty acidity as a result of processing, but it is not enough to be significant.

EFFECT OF ADDED LECITHIN IN CANNED SARDINES

Lecithin has been employed as an antioxidant for oils, fats, and other products and its use was suggested as a possible means of improving the quality of canned sardines. Sardines were packed in 72 one-fourth oil cans; to half of the cans 20 c.c. of plain cottonseed oil was added and to the other half 20 c.c. of cottonseed oil plus one per cent lecithin was

TABLE 1

Effect of Added Lecithin on Volatile Acid Number of Fish

Type of pack	Volatile acid number		
	Processed immediately	Processed after 24 hours in open cans	Processed after 48 hours in open cans
Fish packed in cottonseed oil	12.9	16.0	16.6
Fish packed in cottonseed oil plus 1% lecithin	13.8	17.2	15.7

added. Twelve cans from each lot were sealed and processed immediately, and similar batches were processed after standing periods of 24 and 48 hours. Results of volatile fatty acidity determinations are shown (Table 1).

There was no significant difference between the volatile fatty acidity of the packs containing plain cottonseed oil and those containing the oil with lecithin added. The sardines with plain cottonseed oil added had a slightly stronger odor than those covered with the oil plus lecithin but further studies would be needed before definite conclusions can be drawn.

These cans were held in the field laboratory which was cool at the time of this experiment. This is the probable reason why the volatile fatty acidity was not as high as was found in the 1939 samples held warm in the open cans (Curve 6, Fig. 1).

EFFECT OF PROMPT COOLING AFTER PROCESSING AND PRICKING THE FISH

The sardines in this experiment were packed at a factory where the fish were baked in a hot-air chamber instead of being subjected to a steaming process. Some of the fish were pricked with a fork before baking to see if the loss of oil during the baking process would improve the quality of the canned product. Another lot of fish was baked and chilled immediately after baking to see if such a procedure would have any beneficial effect. Samples from each lot were packed both with and without oil, and control samples from the same batch of fish were also canned. Results

obtained on the canned fish after two weeks' storage period are shown (Table 2).

As measured by volatile fatty acidity content there was no significant difference among any of the packs. Since the fish at the factory where the packs were made are wheeled out into a room where cool sea breezes chill them quite efficiently immediately after baking, any noticeable difference between such fish and those chilled in ice water or by some other method could hardly be expected to be large.

The loss of oil in the punctured fish as measured roughly by the free oil separating from the liquid in the can as compared with that of the control fish indicated that the punctured fish lost seven per cent more oil

TABLE 2

Effect of Puncturing Fish Before Steaming and Chilling After Steaming on Volatile Fatty Acidity

Treatment	Volatile acid number	
	Packed with oil	Packed without oil
Fish punctured before baking	8.4	9.2
Fish chilled immediately after baking	8.1	9.8
Control fish treated in usual manner	8.2	9.8

than the unpunctured. It is possible that more thorough puncturing might cause enough loss of oil so that the flavor would be improved but the application of the procedure to commercial practice might be impractical.

COMPARISON OF CLARK-HILLIG METHOD WITH LOEFFLER METHOD

The Loeffler method for the determination of volatile fatty acidity differs from the Clark-Hillig method in that 25 grams of the ground-up sample of fish instead of an extract of the fish is introduced directly into the distilling flask, and after washing down with a little distilled water the sample is acidified with 50 c.c. of two normal phosphoric acid. The distillation is run at the rate of 100 c.c. per 20 minutes, and it was suggested that titration be made on the first 100-c.c. portion and then on the next 300-c.c. portion.

The special apparatus designed by Loeffler consists of a short-neck Kjeldahl flask, a foam trap, a glass Venturi tube to regulate the rate of distillation, and an ordinary glass water-cooled condenser. The connections between the Venturi tube, foam trap, and Kjeldahl flask were all of ground glass. At the end of the inlet tube from the steam supply is a spray bulb for better dispersion of steam throughout the sample.

For purposes of comparison the distillation rate by the Loeffler method was cut down to coincide with that of the Clark-Hillig procedure, that is, to a rate of 100 c.c. per 30 minutes instead of 20. Also instead of the second portion being 300 c.c. the quantity collected was 100 c.c. to correspond with the recommendation in the Clark-Hillig procedure. On that basis the volatile acid numbers obtained from the same lots of fish were as shown (Table 3).

Volatile acid numbers as determined by the Loeffler procedure were on an average 42 per cent higher than those determined by the Clark and Hillig procedure. Of the eight lots tested the difference was greater in the samples having the lower volatile acid numbers.

Reproducibility of results was much better when the Clark and Hillig procedure was used. Thirty-seven samples were run in duplicate by this method. The average volatile acid number of the high results was 28.2; that of the low 27.6; with an average difference of .6, or 2 per cent.

Seven samples run in duplicate by the Loeffler method had an average volatile acid number of 51.6 for the high figures and 46.3 for the low figures with a difference of 5.3, or 11 per cent. Samples from the same lots determined by the Clark and Hillig method had an average high figure of 35.4 and a low of 35.1 with a difference of .3, or .8 per cent.

TABLE 3

Comparison of Results Obtained by the Loeffler and Clark-Hillig Procedures on the Same Lots of Sardines

Sample	Volatile acid number		
	Loeffler procedure	Clark-Hillig procedure	Per cent increase in Loeffler method
1	27.3	16.7	63
2	83.0	59.7	39
3	18.5	12.9	43
4	56.3	40.2	40
5	39.9	27.0	48
6	25.7	16.9	52
7	66.1	55.5	19
8	75.5	58.5	29
Average	.....	.....	42

The Clark and Hillig apparatus is composed of equipment generally available to any laboratory. The Loeffler apparatus as used in these experiments was made by an expert glass blower according to Loeffler's specifications. However, in his thesis, Loeffler specifies the use of the ordinary 500 c.c. Kjeldahl flask and trap. Such an apparatus was set up and seven determinations were run. The results checked as well as when the special equipment was used, and we believe that the latter is not warranted for a determination such as this.

COMPARISON OF VOLATILE FATTY ACIDITY DEVELOPMENT WITH BACTERIAL GROWTH

Bacterial counts were made on the fish prepared as for the determination of volatile fatty acidity. Suitable dilutions were plated on Petri dishes using tryptone agar and an incubation temperature of 30° C. (86° F.); results are shown (Table 4).

There did not seem to be any definite correlation between bacterial count and volatile fatty acidity; for example, the highest count, 13,000 bacteria per gram, was obtained on the fish held in chloroform-treated sea

water at a temperature of 15.6°C.(60°F.) for 48 hours. The volatile fatty acidity, however, was not much higher than that of the fish with the lowest bacterial count for that day, 55 bacteria per gram, from the fish held in 60° brine at a temperature of 60°F.

In none of the samples was the count unusually high even when the fish were definitely spoiled. If bacteria were responsible for the spoilage, they did not grow profusely under the culturing conditions employed, that is, on tryptone agar in Petri dishes incubated aerobically at 30°C. The other conclusion would be that the development of volatile fatty

TABLE 4

Comparison of Bacterial Count and Volatile Fatty Acidity of Raw Sardines Held Under Various Conditions [1]

Holding medium	Upon receipt at wharf		After holding for 24 hours		After holding for 48 hours		After holding for 72 hours	
	Bacteria per gram	Volatile acid number	Bacteria per gram	Volatile acid number	Bacteria per gram	Volatile acid number	Bacteria per gram	Volatile acid number
Sea water	120	5.4	20	15.6	5,000 [2]	81.4	500	144.7
30° brine (cold)	120	5.4	235	6.0	310	34.4	4,600	105.5
30° brine (warm)	120	5.4	570	8.3	3,500	134.0	..... [3]	..... [3]
60° brine (cold)	120	5.4	30	7.6	55	26.9	150	45.8
60° brine (warm)	120	5.4	320	8.4	540	44.7	..... [3]	..... [3]
Chloroform-treated sea water (cold)	120	5.4	655	7.8	13,000	28.8	100	68.5
Chloroform-treated sea water (warm)	120	5.4	240	26.4	220	89.8	..... [3]	..... [3]
Iced sea water	120	5.4	0	8.0	.....	33.4	400	74.5

[1] The bacteriological counts were made by Professor M. E. Highlands of the University of Maine, who was doing the bacteriological work at the field laboratory. [2] One colony only on plate; count is questionable. [3] Sample discarded after 48 hours.

acidity was due to autolytic action. The data presented here are not extensive enough to justify definite conclusions, yet they do indicate that under some conditions there can be development of volatile fatty acidity in sardines without a substantial increase in bacterial population.

COMPARISON OF ORGANOLEPTIC EXAMINATION WITH VOLATILE ACID NUMBER

Eleven samples of canned sardines were examined organoleptically by six people. The samples were selected to give a wide range of volatile acid numbers as well as different methods of holding; results are shown (Table 5).

The people examining the samples could detect an off-odor in the sample having a volatile acid number of 40 but only two out of six could detect anything wrong with the one having a volatile acid number of 22.8.

One examiner thought he detected an off-odor in sample No. 3, and two rated sample No. 6 as slightly off. Since these two lots had next to the lowest volatile acid numbers of all those tested, we feel justified in stating that only where a majority of the examiners agreed on the rating should it be considered at all definite. In fact one person who examined the samples did not want to give his rating because he would not consider any of the lots as off although he could detect a difference between them.

TABLE 5

Comparison of Organoleptic Examination With Volatile Acid Number of Canned Sardines

Sample No.	Volatile acid number	Organoleptic rating					
		1	2	3	4	5	6
1	16.6	O.K.	O.K.	O.K.	O.K.	O.K.	O.K.
2	59.7	Bad	Slightly off	Slightly off	Off; stale	Bad	Off; bad
3	13.4	O.K.	O.K.	O.K.	Slightly off	O.K.	O.K.
4	22.8	Slightly off	O.K.	O.K.	O.K.	O.K.	Trace; off
5	83.0	Bad	Off	Off	Off; bad	Bad	Off; bad
6	12.8	Slightly off	O.K.	O.K.	O.K.	O.K.	Trace; off
7	40.0	Bad	Slightly off	Slightly off	Off; bad	Off	.....
8	16.7	O.K.	O.K.	O.K.	O.K.	O.K.	Trace; off
9	55.5	Off	Slightly off	Off	Off-odor	Off	.....
10	11.7	O.K.	O.K.	O.K.	O.K.	O.K.	.....
11	78.4	Bad	Slightly off	Slightly off	Slightly off	Off	Off; bad

SUMMARY

As a result of this study we concur with Hillig that volatile fatty acidity may be used as an index of the quality of canned sardines from the standpoint of measuring deteriorative changes which have taken place in the sardines up to the time of canning. The test shows up differences in quality which are not obvious to the average person by organoleptic examination.

Strong brines inhibit the development of volatile fatty acidity. Sardines held in a 60 to 65° salometer brine after equilibrium between the brine and sardines had become established showed practically no increase in volatile fatty acidity even after a holding period of 50 hours. The lowest concentration brine to have any practical inhibiting effect would be a 30° brine (after equilibrium).

High holding temperatures accelerate the development of volatile fatty acidity in sardines.

There was no definite correlation between bacterial count and volatile acid number in the one series of tests made on raw sardines.

Under cannery conditions the factor most likely to cause an appreciable increase in volatile fatty acidity is the holding of sardines in the open can after packing.

In our hands the Clark and Hillig method of determining volatile fatty acidity gave more reproducible results than the Loeffler method, although the latter was much quicker and easier to use. The Loeffler method gave results which were 20 to 60 per cent higher than those obtained using the Clark and Hillig procedure.

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PRESERVING AND GERMICIDAL ACTION OF VARIOUS SUGARS AND ORGANIC ACIDS ON YEASTS AND BACTERIA[1]

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Sugars and organic acids, either natural or added, play an important role in food preservation. The amount and kind of sugar or organic acid to be used for best results frequently is in question. Many food manufacturers consider all acids and sugars equally valuable for preserving purposes. The influence of sugars, acids, and a combination of the two on microörganisms has not been studied extensively. The great expansion of the food industry in recent years and the emphasis being placed upon the microbiological aspects of food indicated the need for such a study.

LITERATURE REVIEW

The literature covering the effect of acids and sugars on microörganisms is reviewed up to 1937 in the paper of Nunheimer and Fabian (1940).

Levine and Fellers (1939) showed that acetic acid was more toxic than either lactic or hydrochloric acid to *Salmonella aertrycke*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. These organisms were inhibited or destroyed at a higher pH value with acetic acid than with lactic or hydrochloric acids. The mold utilized relatively high amounts of lactic acid to develop a growth heavier than that obtained from the acetic acid or the hydrochloric acid series.

Levine and Fellers (1940) found that apart from the indirect effect in altering the hydrogen-ion, the salt and sugar aided the acetic acid but little in its toxic effect on bacteria and yeast. Similarly, the added salt and sugar exerted little, if any, effect on the minimum percentage required for total destruction of these organisms.

In studies on food-poisoning staphylococci Nunheimer and Fabian (1940) found the decreasing order of germicidal action of the acids studied to be acetic > citric > lactic > malic > tartaric > hydrochloric. The decreasing order of antiseptic action was found to be acetic > lactic > citric > malic > tartaric > hydrochloric. They concluded that although the action of the highly dissociated mineral acid is due mainly to the hydrogen-ion concentration, the organic acids exerted a germicidal and antiseptic effect disproportionate to the hydrogen-ion concentration produced. Therefore, it is apparent that the observed effects are due to factors in addition to the hydrogen-ion, presumably either the un-ionized molecule or the anion or both.

They found that dextrose exerted an inhibiting effect in a concentration of 30 to 40 per cent and a germicidal effect at 40 to 60 per cent when

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[1] Summary of thesis presented to faculty of Michigan State College in partial fulfillment of the M.S. degree. Journal Article No. 524 (n.s.).

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using typical strains of food-poisoning staphylococci, whereas, sucrose was less active and a concentration of 50 to 60 per cent was required for inhibition and 60 to 70 per cent for germicidal action. In this work the sugar concentrations were made up by volume.

Nunheimer and Fabian's (1940) results did not agree with those of Levine and Fellers (1940), since the former found that the amount of dextrose which was required to exert a germicidal action could be reduced 50 per cent when used in the presence of one-half the inhibiting concentration of acid. On the other hand, sodium chloride and sucrose could be reduced 30 and 20 per cent, respectively, and still bring about a germicidal effect.

EXPERIMENTAL PROCEDURE

In studying the effects of sugars and acids, the following bacteria and yeasts were used: two thermophiles, *Bacillus calidolactis* and *Bacillus coagulans*, and two mesophiles, *Streptococcus lactis* and *Streptococcus liquefaciens*, of the Hammer collection of Iowa State College; thermophiles isolated from milk powder and raw milk; the yeasts, *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, *Zygosaccharomyces mellis*, and *Torula lactis-condensi* of the Tanner collection of the University of Illinois; and an acid-tolerant yeast isolated from sweet pickles.

The basic medium used for the bacteria and the yeasts had the following composition:

For bacteria	*For yeasts*
15 grams—Bacto peptonized milk	30 grams—Trommers plain malt extract
1.5 grams—Bacto yeast extract	1 gram—NH_4Cl
1.5 grams—Bacto meat extract	1 gram—K_2HPO_4
1,000—water	30 ml.—N/10 citric acid
pH 6.4	1,000 ml.—water
	pH 5.4

The acids were made up in normalities ranging from .1 to 5 and were sterilized by filtration. They were then added in various amounts to sterile test tubes and made up to 10 ml. with sterile broth. The sugars were made up by weight in 100-gram quantities in the respective broths and sterilized by autoclaving at 12 pounds for 15 minutes. They were then pipetted in 10-ml. portions to sterile test tubes. The test tubes were then implanted with either bacteria or yeasts and incubated at the optimum temperature for the organism, and at the end of seven days a subculture of .5 ml. was transferred into broth to determine the presence or absence of growth. At intervals the number of viable organisms was determined by the plate method using Standard milk agar for the bacteria and wort agar for the yeast.

The hydrogen-ion concentration was determined with a Beckman pH meter.

INFLUENCE OF SUGARS ON BACTERIA AND YEASTS

Fructose, dextrose, lactose, and sucrose were used in determining the effect of the sugars upon the organisms. They were added to sterile broth and autoclaved at 12 pounds. All of the sugar concentrations were made up by weight. The highest concentration used for lactose was 30 per cent

and for sucrose 60 per cent, for above those percentages crystallization of the sugars took place in two or three days.

The tubes were inoculated with one or two drops of an actively growing 24-hour culture from five-ml. pipette, depending on the growth in the broth. For the bacteria, the range of the inoculum was such that when a drop or two was added to 10 ml. of sugar solution there were from 50,000 to 150,000 organisms per milliliter, and for the yeast from 100,000 to 200,000 per milliliter.

These tubes were incubated at the optimum temperature for each organism, and at the end of seven days a subculture of .5 ml. was transferred into broth to determine the presence or absence of growth. The preserving percentage was taken as that percentage where there was no growth in the original but there was growth in the subculture broth. The germicidal

TABLE 1

Fructose, Dextrose, Lactose, and Sucrose Required to Exert a Preserving Effect on Bacteria and Yeasts

Organism	Fructose	Dextrose	Lactose	Sucrose
	pct.	*pct.*	*pct.*	*pct.*
Streptococcus lactis	25	30-32.5	None [1]	50-60
Streptococcus liquefaciens	25	30-32.5	None	50-60
Bacillus coagulans	>15 <17.5	25	None	45
Bacillus calidolactis	>15 <17.5	25	None	40-45
Thermophile-milk powder	15	25	None	>42.5 <45
Thermophile-raw milk	>15 <17.5	>25 <27.5	None	42.5-45
Saccharomyces cerevisiae	45	42.5-47.5	None [1]	57.5
Saccharomyces ellipsoideus	42.5-45	45	None	60
Zygosaccharomyces mellis	45-55	47.5-55	None	55-60
Torula lactis-condensi	55	55	None	60
Yeast—pickles	52.5-55	52.5-55	None	60

[1] None means that it was impossible to get a high enough concentration of lactose in solution to be preserving.

percentage was that in which there was no growth in the original tube or in the subcultured broth. All experiments were repeated until four checks had been obtained; the results are shown (Tables 1 and 2).

The order of the preserving action of sugars on the bacteria is fructose > dextrose > sucrose > lactose (Table 1). From 15 to 17.5 per cent fructose was required to exert a preserving action compared with 25 to 32.5 per cent dextrose and 40 to 60 per cent of sucrose. Lactose, with a solubility of only 30 per cent, did not have any preserving action. This table also shows that the preserving percentage varies with the different bacteria; for example, *Streptococcus lactis* and *Streptococcus liquefaciens* required 27.5 per cent fructose while the thermophiles required only 17.5 per cent fructose. This same relationship held true for dextrose where the preserving percentage for the milk streptococci was 30 to 32.5 and for the thermophiles 27.5 per cent, and likewise for sucrose where the former group had a preserving percentage of 60 while the latter group was inhibited at 40 to 45 per cent.

Yeasts are clearly more tolerant than bacteria to the sugars since 45 to 60 per cent was required to bring about a preserving action with yeasts,

whereas with bacteria only 15 to 50 per cent was required (Table 1). The order of activity of the sugars for yeasts differed slightly from that of the bacteria in that fructose and dextrose exerted a preserving action at the same concentration of 45 to 55 per cent, while 60 per cent of sucrose was required to produce the same effect.

The order of the germicidal action of sugars on bacteria was the same as the preserving action with the exception of *Streptococcus lactis* and *Streptococcus liquefaciens*, where sucrose did not exert any germicidal action (Table 2). That the *Streptococcus lactis* and *Streptococcus liquefaciens* are more resistant to sugars than the thermophiles is again demonstrated since the former required a higher concentration of all sugars for a germicidal action than the latter. The table also shows that only fructose and dextrose exerted a germicidal action on all of the yeasts. These sugars

TABLE 2

Fructose, Dextrose, Lactose, and Sucrose Required to Exert a Germicidal Effect on Bacteria and Yeasts

Organism	Fructose	Dextrose	Lactose	Sucrose
	pct.	*pct.*	*pct.*	*pct.*
Streptococcus lactis	27.5	35.0	None [1]	None [1]
Streptococcus liquefaciens	27.5	35.0	None	None
Bacillus coagulans	17.5	27.5	None	47.5
Bacillus calidolactis	17.5	27.5	None	47.5
Thermophile-milk powder	17.5	27.5	None	45.0
Saccharomyces cerevisiae	47.5	50.0	None	60.0
Saccharomyces ellipsoideus	47.5	47.5	None	None
Zygosaccharomyces mellis	60.0	60.0	None	None
Torula lactis-condensi	60.0	60.0	None	None
Yeast—pickles	60.0	60.0	None	None

[1] None means that it was impossible to get a high enough concentration of sugar in solution to be germicidal. Saturated solution of lactose (30%) and sucrose (60%) did not kill in seven days.

required a concentration of 47.5 to 60 per cent to kill all of the yeasts while a concentration of 60 per cent sucrose was germicidal to only one yeast, *Saccharomyces cerevisiae*.

The fact that both dextrose and fructose exerted a germicidal effect at a lower concentration than sucrose and lactose may be explained by the fact that the action of each is, at least in part, due to plasmolysis of the microbial cells. Since this effect depends upon the number of particles present in solution, it is natural that fructose and dextrose, each with a molecular weight of 180, would contain more molecules per unit weight than would sucrose and lactose with molecular weights of 342. Therefore, the activity should tend to decrease as the molecular weight increases.

Since fructose and dextrose have the same molecular weight, the difference in reactivity of these sugars in respect to bacteria must be explained on a chemical rather than a physical basis. The difference in their action on bacteria might be explained by the fact that dextrose is an aldehyde sugar while fructose is a keto sugar. It is a well-known fact in biochemistry that fructose is more reactive than dextrose since many of the chemical tests may be carried out in the cold with the former while heat has to be applied in the case of the latter. Another factor influencing the reactivity

of the two sugars is that fructose has the reactive radical nearer the center of the molecule than dextrose which increases its reactivity as well as its solubility over that of dextrose. These differences in chemical structure apparently account for the chemical as well as the biological differences noted in the two sugars.

INFLUENCE OF ACIDS ON BACTERIA AND YEASTS

In order to study the preserving and germicidal action of the acids on the bacteria and yeasts, the acids were made up as previously indicated.

TABLE 3

Amounts of Acid Necessary to Exert a Preserving Effect on Bacteria and Yeasts

Organism	Acid					
	Acetic		Citric		Lactic	
	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.
Streptococcus lactis	1.5-2.25 (.3N)	.27-.41	1.5-2.25 (.3N)	.315-.473	1.0-1.75 (.2N)	.18-.315
Streptococcus liquefaciens	1.5-2.25 (.2N)	.18-.27	1.5-2.75 (.2N)	.21-.385	1.5(.2N)	.27
Bacillus coagulans	2.1(.1N)	.126	1.7(.1N)	.119	1.1(.1N)	.099
Bacillus calidolactis	1.7(.1N)	.102	1.4(.1N)	.098	1.1(.1N)	.099
Thermophile-milk powder	2.1(.1N)	.126	1.4(.1N)	.098	1.25(.1N)	.113
Thermophile-raw milk	2.9(.1N)	.175	1.8(.1N)	.126	1.25(.1N)	.113
Saccharomyces cerevisiae	1.0-1.5 (.5N)	.3-.45	2.0(1N)-3.0(5N)	1.4-10.5	2.0-3.75 (1N)	1.8-3.38
Saccharomyces ellipsoideus	1.25-1.75 (.5N)	.375-.525	2.0(1N)-1.5(5N)	1.4-5.25	1.75-3.75 (1N)	1.58-3.38
Zygosaccharomyces mellis	1.5(.5N)	.45	2.75(1N) 2.5(5N)	1.93-8.75	1.75-3.25 (1N)	1.58-2.93
Torula lactis-condensi	1.5-1.75 (.5N)	.45-.525	3.0(1N)-2.5(5N)	2.1-8.75	2.0-4.0 (1N)	1.8-3.6
Yeast—pickles	1.25-2.0 (.5N)	.375-.6	2.5(1N)-4.0(5N)	1.75-14	2.25-4.5 (1N)	2.02-4.05

These then were inoculated the same as the sugar solutions and incubated for seven days at the optimum temperature for each organism. At the end of seven days, a subculture of .5 ml. was transferred into suitable broth to determine the presence or absence of growth. The preserving quantity of acid was taken as that quantity where there was no visible growth in the original broth containing the acid but there was growth in the subculture broth containing no acid; results are shown (Tables 3 to 5).

Two methods may be used to determine the preserving and germicidal value of the acids. If the same method is used for evaluating the preserving action of acids as was used for sugars, viz., the number of grams of each acid in 10 ml. of broth, then the order of the preserving value of the

acids for the bacteria is lactic > acetic > citric (Table 3). If, however, the preserving values of the acids are based on pH (Table 5), the order is acetic > citric > lactic.

The order of germicidal value of acids on the bacteria (Table 4) is as follows: lactic > acetic > citric if the number of grams of acid in 10 ml. of broth is used as the basis of comparison, whereas, if the pH is used as the basis, the order is acetic > citric > lactic. The results secured with the acids showed that the same relationship existed between the streptococci and the thermophiles as was found with the sugars. The streptococci were considerably more resistant to acids than were the thermophiles.

On the basis of pH, the order of activity of the acids agrees with the findings of Levine and Fellers (1939) and Nunheimer and Fabian (1940),

TABLE 4

Amounts of Acid Necessary to Exert a Germicidal Effect on Bacteria and Yeasts

Organism	Acid					
	Acetic		Citric		Lactic	
	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.
Streptococcus lactis	2.5(.3N)	.450	2.5(.3N)	0.525	2.0(.2N)	0.360
Streptococcus liquefaciens	2.5(.2N)	.300	3.0(.2N)	0.420	1.75(.2N)	0.315
Bacillus coagulans	2.25(.1N)	.135	2.0(.1N)	0.140	1.25(.1N)	0.113
Bacillus calidolactis	2.0(.1N)	.120	1.5(.1N)	0.105	1.25(.1N)	0.113
Thermophile-milk powder	2.25(.1N)	.135	1.5(.1N)	0.105	1.4(.1N)	0.126
Thermophile-raw milk	3.0(.1N)	.180	1.9(.1N)	0.133	1.4(.1N)	0.126
Saccharomyces cerevisiae	1.75(.5N)	.525	3.5(5N)	12.250	4.0(1N)	3.600
Saccharomyces ellipsoideus	2.0(.5N)	.600	2.0(5N)	7.000	4.0(1N)	3.600
Zygosaccharomyces mellis	1.75(.5N)	.525	3.0(5N)	10.500	3.5(1N)	3.150
Torula lactis-condensi	2.0(.5N)	.600	3.0(5N)	10.500	4.5(1N)	4.050
Yeast—pickles	2.25(.5N)	.675	4.5(5N)	15.750	5.0(1N)	4.500

since they compared the acids in relation to their pH and not on the basis of the amount of acid added.

The order of activity of the acids on the basis of pH is in keeping with the dissociation constants of the acids, since acetic acid with a dissociation constant of 1.86×10^{-5} takes more acid to lower the pH than does citric acid with a dissociation constant of 1.38×10^{-4} or lactic acid with a dissociation constant of 8×10^{-4}. These pH values show that each acid does not depend on the hydrogen-ion activity alone for its action on the bacteria; for example, acetic acid with a lower hydrogen-ion must depend on the un-ionized molecule and the acetate radical as well as the hydrogen-ion of the acid.

The yeasts were more tolerant to acids than the bacteria since an acid strength of .5 to 5 normal was required to bring about a preserving action while for the bacteria a normality of .1 to .3 was required (Table 3). The germicidal quantity of the acids was likewise greater for yeasts than for bacteria (Table 4). However, the order of effectiveness of the acids in the preserving and germicidal range was acetic > lactic > citric for the yeasts. Furthermore, the order remained the same irrespective of whether it was based on pH or the per cent of acid added. There was a considerable dif-

TABLE 5

The pH Values of Acids for Bacteria and Yeasts at Points Where There Is Growth and Preserving and Germicidal Action

Organism	Acetic acid			Citric acid			Lactic acid		
	Growth	Preserving	Germicidal	Growth	Preserving	Germicidal	Growth	Preserving	Germicidal
Streptococcus lactis	4.27	4.12-3.94	3.89	3.96	3.81-3.54	3.40	4.46	3.96-3.55	3.46
Streptococcus liquefaciens	4.31	4.23-4.13	4.09	4.41	4.13-3.63	3.38	3.96	3.63	3.55
Bacillus coagulans	4.52	4.50	4.48	3.50	4.48	4.32	4.40	4.34	4.30
Bacillus calidolactis	4.62	4.60	4.52	4.68	4.52	4.50	4.40	4.34	4.30
Thermophile-milk powder	4.52	4.50	4.48	4.68	4.52	4.50	4.34	4.30	4.20
Thermophile-raw milk	4.37	4.32	4.29	4.49	4.46	4.42	4.34	4.30	4.20
Saccharomyces cerevisiae	4.01	3.79-3.60	3.52	2.28	2.23-1.73	1.70	2.40	2.39-2.21	2.20
Saccharomyces ellipsoideus	3.79	3.68-3.52	3.48	2.28	2.23-1.92	1.82	2.42	2.40-2.21	2.20
Zygosaccharomyces mellis	3.79	3.60	3.52	2.17	2.13-1.79	1.73	2.42	2.40-2.27	2.24
Torula lactis-condensi	3.68	3.60-3.52	3.48	2.13	2.10-1.79	1.73	2.40	2.39-2.20	2.17
Yeast—pickles	3.79	3.68-3.48	3.42	2.20	2.17-1.68	1.65	2.39	2.38-2.17	2.12

ference in the pH needed to kill the yeasts as they were killed at pH 3.5 in the case of acetic acid and at pH 1.75 for citric acid.

INFLUENCE OF MIXTURES OF PRESERVING QUANTITY OF ACIDS WITH SUGARS

A study was next made of a combination of the acids with the sugars to determine the influence of the various combinations of acids and sugars upon the different microörganisms. For this purpose the amount of acid which preserved but did not kill within seven days was arbitrarily chosen as the concentration at which the sugars would be added to the acids. The experiments were carried out by pipetting the preserving amount of the various acids (Table 3) into sterile test tubes and making up to 10 ml. with sterile sugar solutions of varying concentration. These sugar solutions were made to a percentage so that when they were diluted with the acid, the desired concentrations were obtained. These tubes were inoculated and incubated the same as for the acid and sugar experiments.

The percentage of sugar required to bring about a germicidal action on the bacteria was reduced when combined with the preserving quantity of acid (Table 6). For bacteria, the order of effectiveness of the acids in combination with sugars was lactic > acetic > citric. Fructose and dextrose were more effective than either sucrose or lactose in combination with the acids. *Streptococcus lactis* and *Streptococcus liquefaciens* still retained their resistance since a higher percentage of the sugars in combination with the acids was needed to kill them than for the thermophiles. The thermophiles were not able to withstand a combination of fructose or dextrose with any of the acids, as it took less than 2.5 per cent of the sugars to exert a germicidal action. *Bacillus coagulans* and the thermophile isolated from milk powder required from 10 to 20 per cent sucrose in combination with the preserving quantity of citric or acetic acid, while less than 2.5 per cent sucrose was needed to bring about the same germicidal action in combination with the preserving quantity of lactic acid.

It was found for the yeasts that dextrose and fructose in combination with the preserving quantity of the respective acids was germicidal at a lower percentage than either sucrose or lactose in combination with the same amount of acid. In general, it took less dextrose and fructose to bring about a germicidal action with acetic acid than with lactic acid and less with lactic than with citric acid. Sucrose in combination with the preserving quantity of citric acid exerted a germicidal action on only two of the yeasts—*Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus*. No germicidal action was exerted by lactose in combination with any of the preserving quantity of the acids.

The results agree with those of Nunheimer and Fabian (1940) who found that the germicidal amount of dextrose could be reduced by 50 per cent and sodium chloride and sucrose by 30 and 20 per cent, respectively, when used with one-half the inhibiting concentration of acid.

INFLUENCE OF MIXTURES OF ACIDS WITH A CONSTANT PERCENTAGE OF SUGAR

In concluding this work the germicidal percentage of the acids was determined when used in combination with a fixed percentage of the sug-

ars, sucrose and dextrose. The sugar percentage was kept constant at 20 per cent for the bacteria and 35 per cent for the yeasts. The sugar solutions were made up to a percentage so that when they were diluted with the acid, the desired concentrations were obtained. These tubes were

TABLE 6

Fructose, Dextrose, Lactose, and Sucrose Exerting a Germicidal Effect on Bacteria and Yeasts in Presence of Preserving Concentration of Acetic, Citric, and Lactic Acids

Organism	Acid [1]	Acidity [2]	Sucrose	Lactose	Dextrose	Fructose
		ACETIC ACID				
	ml.	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Streptococcus lactis	2.0(.3N)	0.360	20.0	None [3]	12.5	25.0
Streptococcus liquefaciens	2.0(.2N)	0.240	40.0	None	22.5	35.0
Bacillus coagulans	2.1(.1N)	0.126	15.0	None	<2.5	<2.5
Bacillus calidolactis	1.7(.1N)	0.102	<2.5	None	<2.5	<2.5
Thermophile-milk powder	2.1(.1N)	0.126	20.0	None	<2.5	<2.5
Thermophile-raw milk	2.9(.1N)	0.175	<2.5	None	<2.5	<2.5
Saccharomyces cerevisiae	1.25(.5N)	0.375	55.0	None	40.0	40.0
Saccharomyces ellipsoideus	1.5(.5N)	0.450	55.0	None	35.0	25.0
Torula lactis-condensi	1.75(.5N)	0.525	25.0	None	10.0	15.0
Zygosaccharomyces mellis	1.5(.5N)	0.450	45.0	None	35.0	20.0
Yeast—pickles	1.75(.5N)	0.525	30.0	None	15.0	15.0
		CITRIC ACID				
Streptococcus lactis	1.8(.3N)	0.378	30.0	None [3]	17.5	17.5
Streptococcus liquefaciens	2.0(.2N)	0.280	45.0	None	22.5	30.0
Bacillus coagulans	1.7(.1N)	0.119	10.0	<2.5	5.0	<2.5
Bacillus calidolactis	1.4(.1N)	0.098	<2.5	<2.5	<2.5	<2.5
Thermophile-milk powder	1.4(.1N)	0.098	10.0	10.0	<2.5	<2.5
Thermophile-raw milk	1.8(.1N)	0.126	<2.5	5.0	<2.5	<2.5
Saccharomyces cerevisiae	3.5(1N)	2.450	50.0	None	45.0	30.0
Saccharomyces ellipsoideus	3.5(1N)	2.450	40.0	None	25.0	30.0
Torula lactis-condensi	3.5(1N)	2.450	None [4]	None	50.0	50.0
Zygosaccharomyces mellis	3.5(1N)	2.450	None	None	50.0	45.0
Yeast—pickles	3.5(1N)	2.450	None	None	50.0	45.0
		LACTIC ACID				
Streptococcus lactis	1.75(.2N)	0.315	10.0	15.0	<2.5	<2.5
Streptococcus liquefaciens	1.5(.2N)	0.270	30.0	5.0	<2.5	<2.5
Bacillus coagulans	1.1(.1N)	0.099	<2.5	5.0	<2.5	<2.5
Bacillus calidolactis	1.1(.1N)	0.099	<2.5	<2.5	<2.5	<2.5
Thermophile-milk powder	1.25(.1N)	0.113	<2.5	<2.5	<2.5	<2.5
Thermophile-raw milk	1.25(.1N)	0.113	<2.5	<2.5	<2.5	<2.5
Saccharomyces cerevisiae	3.0(1N)	2.700	35.0	None [3]	20.0	20.0
Saccharomyces ellipsoideus	3.0(1N)	2.700	25.0	None	15.0	20.0
Torula lactis-condensi	3.0(1N)	2.700	40.0	None	30.0	40.0
Zygosaccharomyces mellis	2.5(1N)	2.250	35.0	None	25.0	25.0
Yeast—pickles	3.5(1N)	3.150	40.0	None	30.0	30.0

[1] Made up to 10 ml. [2] Calculated. [3] Saturated solution of lactose in combination with acid was not germicidal. [4] Highest possible concentration of sucrose with acid (55%) was not germicidal.

inoculated and incubated the same as for the previous experiments. For these experiments, *Streptococcus lactis* was taken as representative of the mesophiles and *Bacillus coagulans* for the thermophiles.

The percentage of acid required to bring about a germicidal action on the bacteria and yeasts was reduced slightly when combined with a

TABLE 7

Amounts of Acid in Combination With a Constant Percentage of Sugar Exerting a Germicidal Effect on Bacteria and Yeasts

Organism	Acetic acid				Citric acid				Lactic acid			
	Sucrose		Dextrose		Sucrose		Dextrose		Sucrose		Dextrose	
	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.	Ml./10 ml.	Pct.
MADE UP 20% SUGAR												
Streptococcus lactis	2.00(.3N)	.360	1.50(.3N)	.270	2.0(.3N)	0.420	1.5(.3N)	0.315	1.5(.2N)	0.27	1.5(.2N)	0.27
Bacillus coagulans	2.00(.1N)	.120	1.50(.1N)	.090	1.5(.1N)	0.105	1.0(.1N)	0.070	1.0(.1N)	0.09	1.0(.1N)	0.09
MADE UP 35% SUGAR												
Saccharomyces cerevisiae	1.75(.5N)	.525	1.75(.5N)	.525	2.0(5N)	7.000	1.0(5N)	3.500	3.0(1N)	2.70	2.0(1N)	1.80
Saccharomyces ellipsoideus	2.00(.5N)	.600	1.75(.5N)	.525	2.0(5N)	7.000	3.5(1N)	2.450	3.0(1N)	2.70	3.0(1N)	2.70
Torula-lactis-condensi	1.75(.5N)	.525	1.50(.5N)	.450	3.5(5N)	10.500	2.5(5N)	8.750	4.0(1N)	3.60	3.0(1N)	2.70
Zygosaccharomyces mellis	1.75(.5N)	.525	1.75(.5N)	.525	3.0(5N)	10.500	3.0(5N)	10.500	3.0(1N)	2.70	2.5(1N)	2.25
Yeast—pickles	1.75(.5N)	.525	1.75(.5N)	.525	4.5(5N)	15.750	3.5(5N)	12.250	4.5(1N)	4.05	3.5(1N)	3.15

constant percentage of the sugars (Table 7). For the bacteria the order of effectiveness of the acids in combination with the sugars was lactic > acetic > citric and for the yeasts it was acetic > lactic > citric. In general, less acid was needed in combination with dextrose than in combination with sucrose.

The acid is the more important factor in an acid and sugar combination (Tables 6 and 7). Nearly the same amount of acid is required for a germicidal action regardless of whether it is made up in broth or sugar; but once the germicidal concentration is obtained, the percentage of dextrose can be reduced more than can sucrose and still have a germicidal action. For example, .525 per cent acetic acid was required in combination with 35 per cent sucrose or dextrose for a germicidal action on the yeast isolated from pickles (Table 7), but when this acid concentration was kept constant (Table 6) it required only 15 per cent dextrose for a germicidal action as compared with 30 per cent sucrose.

SUMMARY

The order of preserving and germicidal action of the sugars for the bacteria studied is fructose > dextrose > sucrose > lactose. The thermophiles were more susceptible to sugar than *Streptococcus lactis* and *Streptococcus liquefaciens*.

The yeasts were more resistant than bacteria to all the sugars studied. The preserving concentrations of fructose and dextrose were the same for yeasts while for sucrose a five to 15 per cent greater concentration was required. Lactose had no preserving action on the yeasts.

Fructose and dextrose were the only sugars having a germicidal action on all the yeasts. Sucrose was germicidal to only one yeast, *Saccharomyces cerevisiae*.

If the same method is used for evaluating the preserving and germicidal action of acids as was used for sugars, viz., the number of grams of each acid in 10 ml. of broth, then the preserving value of the acids for the bacteria is lactic > acetic > citric. If, however, the preserving and germicidal values are based on pH, the order is acetic > citric > lactic. The streptococci were considerably more resistant to acids than were the thermophiles.

The pH values showed that each acid does not depend on the hydrogen-ion alone for its action on the bacteria but depends partly on the un-ionized molecule or the anion or both.

The yeasts were more tolerant to the acids than the bacteria since an acid strength of .5 to 5 normal was required to bring about a preserving and germicidal action while the bacteria required a normality of .1 to .3. The order of effectiveness of the acids in the preserving and germicidal range was acetic > lactic > citric for the yeasts, irrespective of whether it was based on pH or the per cent of acid added.

For bacteria the order of effectiveness of the acids in combination with sugars was lactic > acetic > citric. Fructose and dextrose were more effective than either sucrose or lactose in combination with the acids.

The thermophiles were not able to withstand a combination of fructose or dextrose with any of the acids, as it took less than 2.5 per cent of the

sugars to exert a germicidal action. *Streptococcus lactis* and *Streptococcus liquefaciens* still retained their greater resistance since higher percentages of the sugars in combination with acids were needed to kill them than for the thermophiles.

It was found for the yeasts that dextrose and fructose in combination with the preserving quantity of the respective acids were germicidal at a lower percentage than either sucrose or lactose in combination with the same amount of acid. In general, it took less dextrose and fructose to bring about a germicidal action with acetic than with lactic and less with lactic than with citric acid.

Sucrose in combination with the preserving quantity of citric acid exerted a germicidal action on only two of the yeasts—*Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus*. No germicidal action was exerted by lactose in combination with any of the preserving quantities of the acids.

In an acid and sugar combination the acid is the more important factor of the two in producing a germicidal effect on microörganisms.

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PRODUCTION OF STAPHYLOCOCCUS ENTEROTOXIN IN CANNED CORN, SALMON, AND OYSTERS [1]

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The conditions under which staphylococci may elaborate enterotoxin in food is an important public-health problem about which little is known. Kelly and Dack (1936) demonstrated that staphylococci could elaborate toxin in sandwiches, in the bread as well as in the meat filler. Dack, Woolpert, Noble, and Halliday (1931) reported the production of enterotoxin in custard-filled cake. Both of these reports were based on the feeding of human volunteers. Jones and Lochhead (1939), using kittens as test animals and diarrhea as a criterion, found enterotoxin in experimentally inoculated fresh corn that was frozen, then defrosted, and incubated at room temperature.

In these studies we have investigated its production in canned foods experimentally inoculated with an enterotoxic staphylococcus, and assayed by feeding or injecting monkeys and by feeding human volunteers.

EXPERIMENTAL PROCEDURE

Corn: Cans of commercially canned, cream-style corn were inoculated with 1 c.c. of an 18-hour culture of an enterotoxic staphylococcus [Strain 161 (4)] and sealed by soldering. In all experiments the corn was incubated for one week at 37.5°C.(99°F.). The cans were then opened and the corn cultured on blood agar to demonstrate growth of staphylococci and absence of contaminants. The contents were mixed with a little water, strained through gauze, and the fluid centrifuged to remove particles of corn, bacteria, etc. One of the following methods was then followed:

(1) Fluid was placed in a dialyzing sac and evaporated by fanning until the equivalent of one can was contained in 50 c.c.

(2) Precipitation with $(NH_4)_2SO_4$ was carried out as with former filtrates, Davison and Dack (1939); the precipitate obtained at full saturation was taken up in water, dialyzed against running water for 24 hours, and evaporated by fanning as in Method 1.

When Preparation 2 was fed before dialyzing, vomiting was caused in every instance, whether or not an enterotoxic staphylococcus was present. This is probably due to the presence of salt in the heavy precipitates obtained.

Since the precipitate was so heavy and the solution turbid, it was felt that feeding would be better than inoculation as used by Davison, Dack, and Cary (1938), as a method of assay, because it requires less rigid control and is less hazardous to the monkey. In eight feedings with Preparation 1, one monkey vomited; and with Preparation 2, none was affected (Table 1).

[1] This study was aided by a grant from the National Canners Association.

The most obvious explanation for negative results in feeding tests is that there is too little toxin present to cause a reaction in the monkeys, in spite of the fact that the equivalent of a whole can of corn was fed to each monkey. Preparation 1, injected intravenously into mice (.5 to 1 c.c.) caused an immediate reaction followed by recovery. Because this reaction would occur within the limits of time for enterotoxin action, it did not seem advisable to use this preparation for intravenous injections. Preparation 2 did not cause any reaction, even in large doses (1 c.c. intravenously). Since mice are not susceptible to enterotoxin, they are injected with all material to be tested intravenously, in order to rule out reactions from the other staphylococcus toxins or poisonous substances—Davison and Dack (1939).

TABLE 1

Assay of Staphylococcus Enterotoxin in Inoculated Canned Foods

Food	Assay method	Untreated foods				Foods precipitated from saturated $(NH_4)_2SO_4$ solution			
		Inoculated (161)		Control		Inoculated (161)		Control	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Corn	Monkey feeding	1	7	....	....	0	6	....	....
	Monkey intravenous	....	....	....	....	2	6	0	3
	Human feeding	1	1	....	....	....	....	....	....
Salmon	Monkey feeding	0	3	....	....	....	....	....	....
	Monkey intravenous	....	....	....	....	0	6	....	....
	Human feeding	0	3	....	....	....	....	....	....
Oysters	Monkey feeding	0	4	....	....	0	2	....	....
	Monkey intravenous	2	6	0	11	1	3	0	1
	Human feeding	1	1	....	....	....	....	....	....

Eight monkeys were injected intravenously with 1 c.c. per kilo of Preparation 2, which had been boiled for 10 minutes. Four samples of corn were used. Control monkeys were similarly injected with material, prepared in the same manner, from cans of corn which had not been inoculated with staphylococci. Two of those receiving inoculated corn vomited in about 30 minutes; the others were not affected. The low percentage of positives obtained by this method rules it out as a practical method of assay but does demonstrate that toxin is present in the inoculated corn, possibly in very small amounts.

Two human volunteers each ate 30 grams of corn which had been inoculated with 1 c.c. of a food-poisoning staphylococcus culture (161) and incubated for one week. One was not affected; the other experienced a typical case of staphylococcus food poisoning. The corn was normal in appearance but the contents tasted definitely sour.

Fresh corn was inoculated with a broth culture of an enterotoxic staphylococcus (161) immediately after cutting from the cob, and samples were incubated for one-half, three, and six hours. The material was passed through filter paper and the filtrate precipitated as before with $(NH_4)_2SO_4$. The precipitate was assayed by intravenous injection into monkeys. Part

of each sample was canned under commercial procedures and the contents later assayed for enterotoxin by feeding of human volunteers.

Eleven monkeys were injected with the dialyzed precipitates of the fresh corn but none was made ill. In view of the former negative results obtained by feeding monkeys with known toxic corn and by injecting precipitates of the unprocessed samples, these methods were not attempted for the corn processed after inoculation and incubation. Two human volunteers ate samples of this corn. It had been inoculated and incubated for three hours before canning, which is a longer period than food is held in the cannery before processing. The corn was normal in odor but was sour (pH 6) and extremely disagreeable in taste. One volunteer ate 60 grams the other 150 grams. Neither was made ill.

In order to determine the role of anaerobiosis in production of enterotoxin in canned goods, the same strain (161) was grown on semisolid agar in Kolle flasks. The flasks were incubated at 37.5°C. in anaerobic jars containing pyrogallic acid and sodium carbonate. Air was pumped out of the jars, CO_2 flushed through, and the jars evacuated again. Complete anaerobic conditions were demonstrated by the lack of pigment in the cultures.

These cultures were strained through gauze and centrifuged for two or three hours and the supernatant fluid was fed to monkeys. Only two of 13 monkeys fed these preparations were made ill. Although this is too small a series to give statistical significance to the figures, it indicates that enterotoxin is not produced as readily under anaerobic conditions as it is aerobically under partial carbon dioxide tension.

Salmon: We attempted to extract enterotoxin from canned salmon experimentally inoculated with an enterotoxic staphylococcus (161) and incubated for three days. The same concentration procedure was followed, that is, precipitation with $(NH_4)_2SO_4$, with the exception that the fluid expressed from the salmon was extracted three times with ether before precipitation in order to remove any fat present, Davison and Dack (1939). Six monkeys were injected intravenously with 1.5 to 2 c.c. per kilo of this preparation without showing any symptoms.

Three monkeys were fed fluid expressed from a whole can of inoculated salmon with no ill effects. Three human volunteers each ate 90 grams of salmon which had been inoculated with staphylococcus (161) and incubated for four days. None was made ill. Although cultures of the salmon showed a heavy growth of staphylococci, the salmon was normal in odor and taste.

Salmon was inoculated heavily with an enterotoxic staphylococcus and incubated for three days in Kolle flasks in 20 per cent CO_2. Fluid from this was fed to four monkeys, but none vomited. However, when salmon was added to the agar media (10 gm. salmon per flask) a good toxin was formed; two monkeys were fed with this and both vomited.

Oysters: The same methods have been used in the study of experimentally inoculated canned oysters. The cans were inoculated with 1 c.c. of a broth culture of an enterotoxic staphylococcus (161) and incubated for three days at 37.5°C. In no case did feeding ground oysters (four feedings) or ammonium sulfate precipitates (two feedings) to monkeys

cause any vomiting. Heated filtrates—98°C.(208.4°F.) for 10 minutes—of these preparations or of the fluids decanted from the cans were injected intravenously into 14 monkeys; two of them vomited. None of the 11 monkeys injected with uninoculated oyster preparations was made ill, with the exception of one monkey which vomited when injected with any of the oyster preparations. It was injected six times, three with inoculated and three with uninoculated, and vomited within five minutes in every case. Each of these preparations was harmless to mice, and the uninoculated preparations did not cause reactions in other monkeys. We cannot explain this monkey's reaction; it was not made ill by feeding oysters or by injecting broth. These reactions are not included in the table.

Two human volunteers drank juice from a can of oysters inoculated with an enterotoxic staphylococcus and incubated for three days. One drank 75 c.c. and the other 100 c.c. The latter experienced a typical attack three and one-half hours following ingestion of the juice.

Canned oysters were used in this investigation because they were reported by Majors, Scherago, and Weaver (1938) to have been responsible for two cases of staphylococcus food poisoning occurring in one family. A staphylococcus [2] was isolated from the open can. Conditions under which the can was kept before analysis were not stated. They demonstrated enterotoxin by the kitten test. We have been unable to produce food poisoning in monkeys with this strain, although six susceptible animals have been fed with three batches of the filtrate. The strain is not hemolytic on blood-agar plates, does not produce a toxin lethal to mice, and does not ferment lactose or hydrolyze gelatin. It reduces nitrates slowly. These properties are entirely inconsistent with those usually found in food-poisoning strains.

We have been unable to confirm the report made by Saslaw, Scherago, and Weaver (1940) that this strain resists heating at 95°C.(203°F.) for 10 minutes. A heavy saline suspension of a 24-hour culture at pH 7.2 did not survive heating at 95°C. for one minute.

DISCUSSION

The probability of food poisoning from canned goods contaminated with staphylococci is slight, since the processing completely destroys these organisms, and since it is unlikely that a leaky can could be contaminated in the cooling bath without obvious spoilage from other bacteria resulting. Experience of the industry is that contamination after processing involves gaseous spoilage. Moreover, the temperatures of standard processing procedures would probably destroy all the enterotoxin formed in the raw foods before canning, according to Davison and Dack (1939). However, we felt it would be advisable to determine whether or not enterotoxin could be produced under optimum conditions in canned foods experimentally inoculated.

Concentration of enterotoxin from foods by precipitation with ammonium sulfate cannot be recommended for routine assay. With experimentally inoculated food under conditions most favorable for the growth of

[2] We are indebted to Dr. Scherago for a culture of this staphylococcus.

staphylococci, we were able to demonstrate enterotoxin in only a small percentage of cases. Intravenous injection of the material, which is a more delicate test than feeding, must be carefully controlled to rule out nonspecific reactions and individual idiosyncrasies such as were encountered in working with the oysters.

The use of human volunteers was deemed necessary owing to the small percentage of positive results obtained by feeding or injecting monkeys. This is undoubtedly due to variation in the susceptibility of the animals as well as to variation in the amount of toxin per can. The three persons fed were not subject to frequent gastrointestinal upsets and displayed no idiosyncrasy to the foods in question.

The factors controlling production of enterotoxin in foods are not known. Growth is not the only necessary requirement, since salmon supported growth of the organisms as well as the other foods. It is not likely that oil in the salmon is responsible for detoxication, Eaton (1938), since the salmon agar medium supports toxin production. Anaerobic conditions may decrease but do not prevent enterotoxin production.

SUMMARY

Certain staphylococci can elaborate enterotoxin in experimentally inoculated cans of corn and of oysters. This was demonstrated by intravenous injection of monkeys and feeding of human volunteers.

We were unable to demonstrate staphylococcus enterotoxin in inoculated canned salmon. The salmon was assayed by intravenous injection into monkeys and by feeding of human volunteers.

Concentration of enterotoxin from foods by precipitation from saturated $(NH_4)_2SO_4$ solution can be accomplished, but it does not give a high enough percentage of positive results to be used as a practical routine method of assay.

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RIBOFLAVIN CONTENT OF SOME COMMON FOODS[1]

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Riboflavin is now known to be an essential constituent of the diet of man as shown by the work of Sebrell and Butler (1938); Oden, Oden, and Sebrell (1939); and Sydenstricker, Geeslin, Templeton, and Weaver (1939). Many of the acute symptoms indicating an inadequate intake of this factor have been identified in the investigations carried out by Sebrell and Butler (1939); Jolliffe, Fein, and Rosenblum (1939); Kruse, Sydenstricker, Sebrell, and Cleckley (1940); and Sydenstricker, Sebrell, Cleckley, and Kruse (1940). Studies recently reported by Spies, Bean, Vilter, and Huff (1940) indicate that the use of diets containing insufficient riboflavin for good nutrition may be much more common than is generally realized. To help correct this situation it is desirable to extend our knowledge of the occurrence of riboflavin in foods. The present study was undertaken to obtain information on the riboflavin content of some of our more common foods including representatives from the different food groups.

EXPERIMENTAL PROCEDURE

Method of Assay: The rat-growth method was used. Young rats, weaned when 28 days old and weighing between 45 and 55 grams, were given a diet made up of vitamin-free casein, 18 per cent; starch, 66 per cent; corn oil, 8 per cent; cod liver oil, 2 per cent; desiccated rice polish extract,[2] 2 per cent; and salts (U. S. P. XI, second supplement, p. 133), 4 per cent. This diet contained no more than traces of riboflavin, and when supplemented with riboflavin, supported normal growth for periods in excess of eight weeks. The animals were housed separately in cages that had raised bottoms of large mesh screening; each animal had access to the basal diet and distilled water *ad libitum*. At the end of one week they were weighed and outfitted with harnesses as designed by Page (1932) to prevent coprophagy. A record of the weight was made again at the end of the second week and daily thereafter until a majority in the litter showed constant or declining weight when supplemental feeding of the test material and the standard riboflavin solution was begun.

The general plan for the assay involved selecting three quantities of the test material and one of the standard of reference for feeding. The quantity of the standard considered to give the most satisfactory results with the test animals available for these assays was three micrograms per

[1] The work here published was carried out through the coöperation of the Bureau of Home Economics of the United States Department of Agriculture and the Department of Chemistry of Columbia University. The author is indebted to Professor H. C. Sherman of the latter department for suggestions in the course of the work.

[2] A commercial desiccated extract of rice polish prepared under controlled conditions. This product is guaranteed by the manufacturers as a source of vitamin B_1 substantially free of riboflavin and is described as an efficient source of other water-soluble vitamins.

rat per day. This was given in the form of an acidified alcohol-water solution from a graduated syringe. The three quantities of test material were chosen on the basis that one would supply approximately three micrograms of riboflavin, another less than three but more than one microgram, and the third quantity more than four but less than eight micrograms. These three levels of test material and the three micrograms of riboflavin were allotted to litter-mate animals of comparable weight. The supplemental feedings were given three times per week in quantities sufficient for two days. The general plan was to use a sufficient number of litters to give a total of six to eight animals in each of the four assay groups. The animals were weighed at the end of each week from the time supplemental feeding was started until four weighings had been made.

The riboflavin content of a food was estimated from the results by first finding the total gain in weight made by each animal during a specified period from the time supplemental feeding was begun. The average gains in weight for the three assay groups on the test material were then plotted against the quantity of test material fed as a daily supplement; a smooth curve, the curve of response, was drawn through these points. The quantity of test material corresponding to the average gain in weight shown by the group of animals given three micrograms of riboflavin was derived from this curve, and the riboflavin content of the food was calculated on the basis that this quantity contained three micrograms. The final value was usually arrived at by considering the estimate made on the basis of the gains in weight during the first three weeks of the test period as well as the estimate made from the gains in weight at the end of four weeks.

Food Samples: With the exception of the samples described as grain-fed and grass-fed beef the various foods studied were sampled by purchasing small quantities periodically in the local retail stores. This meant that during the period necessary to complete the assay of a particular food a number of samples were used which may have differed considerably in their history. Since, under such conditions, there is no way of judging what relation the final estimate of riboflavin content has to the actual content of the several samples used, every effort was made to give the animals used in the assay portions from all samples of the food. When it was not possible to do this and different values were derived from the results with different samples of a food, the separate estimates are given. No attempt was made to determine the part of such differences ascribable to sample variation apart from experimental error.

In purchasing the food samples care was taken to select material of good quality but not necessarily the most expensive kind or the highest quality. If the quantity purchased at a given time was sufficient for several feedings, the carryover was stored to avoid loss of weight or other changes that might affect the concentration of riboflavin. Perishable material was kept in the refrigerator in a closed container. During the preparation of the samples and the weighing and feeding of the supplements care was taken to avoid loss of riboflavin from exposure to light.

This report covers results obtained with 22 separate food items including milk, cheese, and eggs; meat and fish; bleached and green leafy

vegetables; as well as legumes, root vegetables, and cereals. So far no fruits have been investigated since representative types of fruits have already been studied by Lanford, Finkelstein, and Sherman (1941) and their values reported.

DESCRIPTIONS OF FOODS TESTED AND METHOD OF SAMPLING

Dairy Products and Eggs: Milk, whole, pasteurized—a pint bottle of grade-B milk was purchased each day supplements were fed, always from the same distributor, one who sells a large volume of milk at retail in New York City. Immediately before the supplements were to be measured out, the milk was poured back and forth from one container to another to insure uniform distribution of the cream.

Cheese, American Cheddar—(A) the cheese used was a single brand made in Wisconsin. It was a mild cheese sold in half-pound bricks wrapped in metal foil. All samples were purchased from the same store, and each sample served for one week. The unused portion, tightly wrapped in the original cover, was kept in the refrigerator. (B) All samples were purchased in a single store and were obtained as slices from a large cake sold as "store" cheese.

Eggs, whole—(A) all samples were purchased in a single store and were from a lot described as "grade A, New Jersey White Leghorn." (B) All samples were purchased in a single store belonging to a well-known chain grocery. The eggs were described as fresh pullet eggs, mixed brown and white. Usually a purchase consisted of six eggs. On each day of feeding two eggs were prepared by keeping them in water just below the boiling point for 10 to 15 minutes. They were then cooled in running water, and after the shells were removed, were put through a fine-meshed sieve and thoroughly mixed.

Meat and Fish: Beef muscle, top round—all samples were purchased in a single store and were from cuts marked certified. A fresh sample was purchased each day supplements were fed. Only the lean portion free from visible fat was used.

Beef muscle—two special samples were supplied by the Bureau of Animal Industry, U. S. Department of Agriculture. One sample was from grain-fed cattle and was graded "high commercial"; the other was from grass-fed cattle and was graded "low good." In preparing the samples in the Bureau of Animal Industry, eye muscle was removed from the 9-11 rib cut; approximately equal amounts of tissue from ten animals were ground together and packed in bottles. The bottles were then stored in a freezer room at —17.8°C.(0°F.). An amount of this material sufficient for the assays came from Washington, D. C., packed in dry ice, and was stored in the freezing compartment of a refrigerator for the duration of the assay.

Pork muscle, lean of chops—all samples were purchased from a single store and were described as from young, corn-fed hogs. A fresh sample was purchased each day supplements were to be weighed and only the lean portion was used.

Salmon, red, canned — one can was purchased each week and was opened just before the first sample was to be taken. The solid meat was

lifted out of the can and held suspended until there was no dripping. The skin and bone were then removed and the remainder thoroughly mixed. The portion kept for successive tests was stored in a tightly covered glass container in the refrigerator. The contents were thoroughly mixed before each sample was taken.

Vegetables, Green Leafy: Beans, green, snap, young—a fresh sample was purchased each time supplements were fed. The quantities for the supplements were obtained by cutting crosswise sections with a view to obtaining a natural proportion of whole bean and pod. As the assay covered a considerable period of time, several varieties of the bean may have been represented in the various samples used.

Broccoli—each purchase consisted of one bunch which generally served for the feedings made during a week. The flower portion included the buds and the stems down to the main plant stalk. The "leaves" used in the assay were the thin portions left after trimming out the thick rib sections.[3]

Cabbage, head, pointed type—heads weighing three to four pounds were purchased and usually served for two successive feedings. The outer leaves were naturally much greener than the inner leaves, and in preparing the supplements effort was made to give a proportionate share of inner and outer leaves to each test animal. The main stem was discarded from the leaves and the innermost leaves were not used.

Endive (escarole)—a single plant was purchased at a time and usually served for one week. Most of the samples had fairly large green leaves and the head was almost entirely green. Samples were obtained by first removing the thick mid stem from a leaf and then cutting sections crosswise. The portion kept for successive feedings was stored in the refrigerator in a tightly closed glass jar.

Lettuce, Iceberg, or New York type—tightly packed heads showing very little green color were used. Treatment for sampling was the same as for escarole and cabbage.

Peas, green, young—each sample was purchased immediately before it was to be used. Whole peas were weighed, and when it was necessary to use a part of one to complete the portion the division was made so as to obtain as nearly as possible a representative sample.

Pepper (red pepper), green—the whole pepper was sampled by removing the seeds and the core and cutting sections from the stem end to the tip.

Spinach, leaf blades only—a sample was purchased for each day of feeding. As soon as it was brought to the laboratory it was washed and left to freshen. Just before the portions were to be weighed, whole leaves were removed by cutting the stem at the base of the leaf, the water was shaken off and the leaves taken to the balance. Each separate leaf was freed from water adhering to it immediately before weighing a portion which was obtained by cutting sections crosswise. The small crown leaves and the stems were not used.

[3] The leaves are perhaps not often retained when broccoli is prepared for cooking. Like other green leaves they are rich in vitamin A and were studied here with the idea of obtaining further evidence of the high nutritive value of broccoli.

Vegetables, Roots and Tubers: Beets, red — the samples were from young beets having fresh leaves when purchased. The beets were washed, and the rootlets and leaves including about one-quarter inch of the top of the beet were removed. Samples were taken by cutting wedges lengthwise of the root.

Carrots — young, bunch carrots were used. After washing, approximately one-quarter inch of the top and one inch of the tip were discarded. The remainder was quarter-sectioned by splitting lengthwise and samples were taken by cutting these strips crosswise. Sections from top, bottom, and middle were included in each portion.

Potatoes, white, stored, with peel—the samples used were purchased during the last of February and the first of March. Medium-sized tubers were used. A one-half inch slice was first removed from the bud end and samples were taken by slicing the remainder crosswise. Wedge-shaped pieces were used to complete the sample to the specified weight.

Sweet potatoes—the potatoes were purchased in one- and two-pound lots as needed. When portions were to be weighed the potatoes to be used were washed and dried. The skins were not removed. About one inch of the end of the potato was discarded and portions were then obtained from slices about one-quarter inch thick cut crosswise of the root. Wedge-shaped pieces from a slice were used to complete the weight of a portion when a fraction of a slice was needed. When the portion of potato had been weighed it was cut into small pieces and placed in a glass ointment jar with a small quantity of water. The jar was closed by putting the cover on loosely and kept in a steam bath for 15 minutes.

Legumes, Dried: Beans, navy, dried—two pounds of beans were purchased for the assay. After all foreign material and broken and unsound beans had been removed the sample was pulverized by grinding twice in a laboratory mill. A portion for feeding was prepared by placing the weighed amount of material in a glass ointment jar with sufficient water to give a thin paste and then keeping the closed jar in a steamer for 15 minutes.

Cereals: Corn meal—six cartons of granulated yellow corn meal were purchased, each from a different store. This product consisted almost entirely of endosperm. After the contents of the six cartons were mixed the sample was pulverized in a laboratory mill and stored in glass jars. Portions sufficient for one week's feeding were weighed out at one time.

Oatmeal — commercial rolled oats (quick cooking) were used. The sample was obtained and treated in the same way as the sample of corn meal.

Wheat—the sample used was taken from the laboratory supply of ground whole wheat. This wheat, described as hard winter, is purchased as needed and is ground in the laboratory.

RESULTS

The values obtained for the riboflavin content of the 20 food items studied are summarized (Table 1); the inclusion of more than one value for a single food item indicates that more than one set of samples was

TABLE 1

Riboflavin Content of Some Common Foods

Food	Riboflavin	Investigator
	μgm./100 gm.	
Dairy products and eggs		
Milk, pasteurized, market	214[1]	Present study
Cows on dairy ration	200	Kunerth, *et al.* (1937)
Cows on good pasture	270	Kunerth, *et al.* (1937)
	127[1]	Murthy (1937)
	100-150[1]	Neuweiler (1937)
	176-255[1]	Whitnah, *et al.* (1937)
	200-260[1]	Whitnah, *et al.* (1937)
	100-130[1]	Baars (1938)
	148-180[1]	Emmerie (1938)
Herd cows, winter rations	200	Kramer, *et al.* (1938)
Holstein and Jersey cows, 5th day of lactation	330; 370	Kramer, *et al.* (1938)
Holstein and Jersey cows, 30th day of lactation	170; 240	Kramer, *et al.* (1938)
	780[1]	Lindholm (1938)
	275	Lunde, *et al.* (1938)
Different breeds of cows on winter rations	117-173	Whitnah, *et al.* (1938)
	80; 90	Wilson and Roy (1938)
	120-340[1]	Hand (1939)
	60-342	Hand and Sharp (1939)
	100-150[1]	Houston, *et al.* (1939)
Different breeds of cows on pasture and on winter rations	160-280	Kramer, *et al.* (1939)
	240	Lunde, *et al.* 1939b)
	169; 200	Snell and Strong (1939)
	150-250	Snell and Strong (1939)
Raw, February-July	96-159[1]	Houston, *et al.* (1940a)
Pasteurized or sterilized, February-July	107-166[1]	Houston, *et al.* (1940a)
Cows on dry feed	110[1]	Houston, *et al.* (1940b)
Cows on pasture	150[1]	Houston, *et al.* (1940b)
Cows on pasture	150-240[1]	Johnson, Maynard, and Loosli (1941)
Cows on winter ration	150-230[1]	Johnson, Maynard, and Loosli (1941)
Cows on herd ration with molasses and yeast	150-260[1]	Johnson, Maynard, and Loosli (1941)
Cheese, American Cheddar		
(A)	600; 545	Present study
(B)	545	Present study
Eggs, whole		
(A) Hen eggs	306	Present study
(B) Pullet eggs	320	Present study
	.....[2]	Hunt, Winter, and Bethke (1939)
From farm flocks	207; 248	Norris and Bauernfeind (1940)
	190-230	Snell and Quarles (1941)
Meat and fish		
Beef, muscle, top round	214; 222	Present study
Rib, eye muscle, grain-fed cattle	140	Present study
Rib, eye muscle, grass-fed cattle	135	Present study
	180-190	Baars (1938)
	350	Lunde, *et al.* (1938)
	190	Mickelsen, *et al.* (1939)
	350; 240	Waisman and Elvehjem (1941)

[1] Micrograms per 100 milliliters. [2] Proportional to quantity riboflavin in the diet of the hen.

TABLE 1 *(Continued)*

Riboflavin Content of Some Common Foods

Food	Riboflavin	Investigator
	μgm./100 gm.	
Pork, muscle, lean of chop	216; 218	Present study
	240	Lunde, *et al.* (1938)
Ham	200-270	Mickelsen, *et al.* (1939)
Ham, boiled	260; 280	
Ham, smoked	230-290	
Ham, tender	200; 240	
Loin	200-270	
Pigs on low-flavin diet	140; 170	Ittner and Hughes (1941)
Pigs on high-flavin diet	250; 260	
Pigs on normal grain ration	210	
Salmon, red, canned	250; 195	Present study
Vegetables, green, leafy		
Beans, green, snap, young	115; 107	Present study
	93	Hodson (1940)
	161	Conner and Straub (1941a)
Broccoli, leaves	460	Present study
Flower	250	Present study
	210; 226	Hodson (1940)
Frozen	176	Conner and Straub (1941a)
Cabbage, green, winter market	38	Present study
	215	Murthy (1937)
	25; 32	Wilson and Roy (1938)
	40-50	Flavier and Genevois (1939)
	44; 66	Hodson (1940)
Endive (escarole), very green	214; 187	Present study
Lettuce, Iceberg or New York type	Less than 25	Present study
	128; 116	Wilson and Roy (1938)
Inner leaves	60-80	Flavier and Genevois (1939)
Outer leaves	30-80	
Head	48	Hodson (1940)
Leaf, stemmy	96	
Leaf	71	
Peas, green, fresh, young	200	Present study
	280	Murthy (1937)
	87; 10	Wilson and Roy (1938)
	30-50	Flavier and Genevois (1939)
	180	Lunde, *et al.* (1939b)
	131; 154	Hodson (1940)
	210[a]	Fellers, *et al.* (1940)
Frozen	190	Conner and Straub (1941a)
Pepper (red pepper), green	About 30	Present study
Green chillies	180	Murthy (1937)
	138	Hodson (1940)
Spinach, leaf blades only	371; 424	Present study
	124; 89	Wilson and Roy (1938)
	335-370	Lunde, *et al.* (1939b)
	159; 160	Hodson (1940)
	436[a]	Fellers, *et al.* (1940)
	117-147	Conner and Straub (1941a)

[a] Derived from Bourquin-Sherman values.

TABLE 1 *(Concluded)*

Riboflavin Content of Some Common Foods

Food	Riboflavin	Investigator
	μgm./100 gm.	
Vegetables, roots and tubers		
Beets, red	71	Present study
	85	Wilson and Roy (1938)
	23	Hodson (1940)
Carrots	62	Present study
	Trace	Murthy (1937)
	18	Wilson and Roy (1938)
	67	Hodson (1940)
Potatoes, white, with peel	25-30	Present study
	43	Murthy (1937)
	40-60	Baars (1938)
	48	Wilson and Roy (1938)
	44; 55	Hodson (1940)
Sweet potatoes	50	Present study
	110-220	Baars (1938)
	44; 70	Wilson and Roy (1938)
	68; 70	Hodson (1940)
Legumes, dried		
Beans, navy, dried	300; 324	Present study
Cereals		
Corn, yellow corn meal, commercial	Less than 33	Present study
White	309	Murthy (1937)
Yellow	49; 36	Wilson and Roy (1938)
Meal	20	Hodson and Norris (1939)
Granulated meal	60	Hodson and Norris (1939)
Yellow corn	83-202	Conner and Straub (1941a)
Yellow corn meal	50-100	Conner and Straub (1941b)
Oats, rolled oats, commercial	100	Present study
Rolled oats	110	Hodson and Norris (1939)
Groats	140	Hodson and Norris (1939)
Wheat, whole		
Sample No. 1	100	Present study
Sample No. 2	80-85	Present study
	119	Murthy (1937)
	37; 40	Wilson and Roy (1938)
	80	Hodson and Norris (1939)
	140	Lunde, *et al.* (1939a)
	89-203	Conner and Straub (1941a)
Hard	117	Conner and Straub (1941b)
Soft	107	Conner and Straub (1941b)

used to complete the assay and that the estimates made on this basis did not coincide. Further study is required in each case to determine whether this was due to sample variation or to error of assay.

Values for the same food items reported by other investigators are also given (Table 1). These include values determined by the *in vitro* and the microbiological methods as well as the rat-growth technique. With the exception of three values which are noted no values have been included that were not determined directly in terms of pure riboflavin.

DISCUSSION

In judging the significance of such values as those in Table 1 certain general considerations should be taken into account.

When the assay procedure is carried out as described in this report a daily supplement of one microgram of riboflavin is the least amount that will promote a significant growth response. The range of values for daily supplements of riboflavin within which the average gains in weight of comparable groups of assay rats are proportional to riboflavin intake is from one to eight micrograms. The least difference in riboflavin value that may be determined satisfactorily with assay groups of not more than six to eight rats each is one microgram. The level of riboflavin most satisfactory for purposes of comparison in making assays is three micrograms.

The type of food material being assayed also has an influence on the degree of precision of the assay. If the food material is of high caloric value it is usually not feasible to feed daily supplements of greater amount than four grams since with larger amounts the intake of basal diet may be so small that some other nutritive essential becomes the first limiting factor for growth. This means that in assays on foods of this type containing less than 25 to 30 micrograms of riboflavin per 100 grams positive results are probably no more than indicative of the presence of riboflavin. It is doubtful if foods containing more than 30 micrograms but less than 100 micrograms per 100 grams can be assayed by the rat-growth method with any appreciable degree of precision unless large numbers of animals are used. Foods having a high water content can be fed at somewhat higher levels—six to eight grams per rat per day—than is possible with those that are more concentrated. Foods of this type having a concentration of riboflavin as low as 50 micrograms per 100 grams may be assayed with a fair degree of precision if a sufficient number of animals is used.

On the basis of these considerations it is suggested that the significance of riboflavin values for foods should be appraised in relation to the type of food material as well as on the basis of other factors known to influence the degree of precision of the assay.

SUMMARY

Samples of representative foods from the different food groups have been assayed for riboflavin by the rat-growth method. These included milk, eggs, one type of cheese, muscle meat of beef and pig and fish, leafy vegetables both green and bleached, a seed pod, three root vegetables and one tuber, a fresh and a dried legume, and three cereal grains.

The highest value for riboflavin content was obtained with Cheddar cheese. This is as would be expected since the riboflavin of milk, which is a rich source of this factor, is associated with the protein. The dried legume was also an excellent source. Milk, beef muscle, pork muscle, and fish (salmon) muscle each assayed more than 200 micrograms per 100 grams. Different values were obtained for samples of eggs purchased at different times which is in accordance with the findings of others regarding variation in the riboflavin content of eggs. Among the green leafy vegetables spinach and the leaves of broccoli gave very high values in parallel with their greenness. They were intermediate between the values

for cheese and those for the dried legume. The values for green string beans, fresh green peas, and endive are similar to those for milk and muscle meats. The less green leaves of cabbage and lettuce gave very low values. Green pepper presents an anomaly since the value obtained for it is similar to that for cabbage and lettuce although in degree of greenness it is more liks spinach and broccoli. Roots and tubers seem to be uniformly low and cereals are intermediate. The very low value for corn meal is probably due to the fact that during processing the larger part of the germ and outer covering of the grain were removed.

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STAPHYLOCOCCI AND SALMONELLA CONTROL IN FOODS

III. EFFECT OF CHOCOLATE AND COCOA FILLINGS ON INHIBITING GROWTH OF STAPHYLOCOCCI

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Since there are no food-illness cases (to the authors' knowledge) alleged to be due to bakery chocolate or cocoa fillings, the inhibiting effect of these fillings on the growth of staphylococci has been investigated.

EXPERIMENTAL PROCEDURE

The chocolate and cocoa fillings were made, using the following basic formula:

Water	1 qt.	Starch	3½ oz.
Sugar	1 lb.	Butter	1 oz.
Salt	⅛ oz.	Chocolate	4 oz.
Egg yolks	5 oz.		

The fillings were made by bringing three-fourths of the water, the sugar, and the salt to a boil. Then the rest of the water and starch were added, and the mixture was cooked until it was clear. At this point the fire was turned off, the egg yolks, butter, and chocolate were added, and all ingredients mixed thoroughly. The changes made in the mix were variations in the quantity and kind of chocolate and variations in the quantity and kind of cocoa when used in place of chocolate. Mixes specifying a certain quantity of cocoa did not contain chocolate and vice versa. Also, in some mixes the eggs were left out.

A standard custard of the following formula was run with each experment in order to have a standard for comparison.

Water	1 qt.	Powdered whole milk	4 oz.
Sugar	12 oz.	Eggs	4 oz.
Salt	⅛ oz.	Cornstarch	3 oz.

Three-fourths of the water, eight ounces of sugar, the salt, and the powdered whole milk were brought to a boil. The cornstarch and the remaining four ounces of sugar were blended together and added to the remaining one-fourth quart of water and the eggs; these ingredients were whipped till a smooth, free-of-lump mixture was obtained. This was then added to the above mixture, brought to a second boil, and retained at that temperature for two minutes.

The same culture of *Staphylococcus aureus* as was used previously by Cathcart, Ryberg, and Merz (1941) was used in this work. The bacteriological procedure was handled in the same manner as that reported by Ryberg and Cathcart (1941), except that all of the samples were autoclaved before they were inoculated.

TABLE 1

Effect of Chocolate and Cocoa Fillings on Growth of Staphylococcus aureus

Series	Type of filling	Organisms inoculated per gram of custard	Organisms per gram after 24 hours' incubation	Ratio of organisms found to those inoculated	pH
1	Standard custard	4,780,000	104,000,000	21.80	6.47
	0 oz. natural chocolate No. 1[1]	4,700,000	12,900,000	2.70	6.39
	2 oz. natural chocolate No. 1	4,420,000	60,000,000	13.60	5.69
	4 oz. natural chocolate No. 1	4,320,000	600,000	0.14	5.50
	4 oz. natural chocolate No. 1, no eggs[2]	4,700,000	160,000	0.03	5.35
	8 oz. natural chocolate No. 1	3,820,000	3,300,000	0.86	5.39
2	Standard custard	83,790	910,000,000	10,860.00	5.84
	0 oz. natural chocolate No. 1[1]	82,000	236,000,000	2,878.00	5.09
	4 oz. natural chocolate No. 1, no eggs	78,560	6,000	0.08	5.31
	2 oz. natural chocolate No. 1	73,550	16,700,000	227.00	5.07
	4 oz. natural chocolate No. 1	74,050	13,750,000	186.00	5.10
	8 oz. natural chocolate No. 1	69,840	1,930,000	27.60	5.00
3	Standard custard	65,900	710,000,000	10,775.00	6.10
	4 oz. natural chocolate No. 1	57,180	11,400,000	199.00	5.41
	4 oz. natural chocolate No. 1, no eggs	71,310	49,000	0.69	5.13
	3 oz. natural cocoa No. 1, no eggs	72,150	53,500	0.74	5.20
	3 oz. natural cocoa No. 1	65,900	3,450,000	52.40	5.40
	3 oz. medium Dutched cocoa No.2	64,520	61,500,000	953.00	6.39
4	Standard custard	20,090	1,090,000,000	54,266.00	6.07
	3 oz. cocoa No. 3, 20% fat, no eggs (blend of natural and Dutched)	22,350	293,000	13.10	6.90
	3 oz. natural cocoa No. 4, 32% fat, no eggs	21,370	48,000	2.20	6.25
	3 oz. natural cocoa No. 5, 22% fat, no eggs	21,620	22,500	1.04	5.19
	3 oz. natural cocoa No. 6, 26% fat, no eggs	21,150	21,600	1.02	6.10
	3 oz. natural cocoa No. 7, no eggs	21,150	23,000	1.09	5.45
5	Standard custard	11,740	78,000,000	6,647.00	6.49
	4 oz. natural chocolate No. 2, no eggs	11,740	1,000	0.09	5.70
	4 oz. natural chocolate No. 3, 53% fat, no eggs	11,210	4,000	0.36	5.67
	4 oz. natural chocolate No. 4, 53% fat, no eggs	11,600	4,000	0.34	5.31
	4 oz. natural chocolate No. 5, 53% fat, no eggs	10,840	4,000	0.37	5.40
6	Standard custard	11,520	12,500,000	1,083.00	6.60
	3 oz. natural cocoa No. 8	11,520	3,200	0.28	6.00
	3 oz. natural cocoa No. 8, no eggs	12,910	130	0.01	5.98
	4 oz. natural cocoa No. 1, no eggs	11,980	6,500	0.54	5.65
	4 oz. natural cocoa No. 1, no eggs	13,320	160	0.01	5.49

[1] Filling contained sugar, egg yolks, salt, starch, butter and water, i.e., same basic formula as other chocolate and cocoa fillings. [2] Unless otherwise specified, eggs were used in all cooking experiments.

RESULTS AND DISCUSSION

The fillings in each series were treated simultaneously, and owing to variations in the inoculums, series-to-series comparisons must be made on a series basis. The slight variation in the inoculum from filling to filling within a series is due to the variations in the weight of each individual filling. The ratio of the organisms found after incubation to the number inoculated is given to facilitate comparisons.

These data (Table 1) show that the fillings made with natural chocolate or cocoa are effective in inhibiting the growth of *Staphylococcus aureus*, in comparison with a standard custard. The degree of inhibition to some extent is influenced by the number of organisms inoculated; for instance, the fillings in Series 2 show more growth on incubation than the same fillings do in Series 1, when a much greater inoculum was used.

There are probably several reasons why the chocolate and cocoa fillings made with natural chocolate and cocoa are effective in inhibiting the growth of *Staphylococcus aureus*. First, the inhibition is undoubtedly due to the chocolate or cocoa itself or to some natural substance which it contains. Second, the inhibition is due to a lower pH of these fillings. In general, the fillings made with natural chocolate or cocoa have a lower pH than the standard custard. The inhibitory effect shown by decreasing the pH of the medium has been demonstrated before in this series of papers, Ryberg and Cathcart (1941), and by other workers. Although the pH showed some variation from series to series, the medium Dutched cocoa[1] filling in Series 3 and the mixture of natural and Dutched cocoa filling in Series 4 show that an increase of pH of the filling decreases to some extent the inhibitory action of the cocoa. Yet, these later fillings demonstrated considerable inhibiting action, undoubtedly owing to the fact that the cocoa itself contains a substance or a mixture of substances that contributes greatly to this action. Third, the fact that milk was not used in the fillings undoubtedly makes them more effective. As shown before by Ryberg and Cathcart (1941) milk, when added to a filling, increases the pH of the mixture and thus would tend to decrease slightly the effectiveness of the chocolate and cocoa. Milk was not used in the fillings since it is not generally an ingredient included in such fillings when they are made by the food manufacturer.

Consideration of all the experiments shows that the fat content of the chocolate was not a factor in this inhibiting action, for three ounces of cocoa (supplying solids-not-fat approximating the solids-not-fat in four ounces of chocolate) showed about the same effectiveness as four ounces of chocolate; yet, there was considerably less fat in the filling made from the cocoa. This is also demonstrated to some extent by Series 4, where the natural cocoas used showed a considerable difference in fat content.

The effectiveness of chocolate and cocoa in inhibiting the growth of *Staphylococcus aureus* was greatly increased by eliminating eggs from the formula. The fillings which did not contain eggs are indicated (Table 1). In only two cases did these fillings show any increase in the number of organisms on incubation for 24 hours. These are the first two cocoa fill-

[1] Dutched cocoa is cocoa to which alkali has been added.

ings in Series 4. The first of these is not a natural cocoa but a blend of natural and Dutched cocoa.

The increase in the inhibitory action of a filling, when the eggs were not included, is probably due to the fact that less nutrient material was available for growth of the organisms. These fillings without eggs would be entirely satisfactory from the commercial standpoint, for flavor tests on fillings (containing four ounces of chocolate or three ounces of cocoa) with and without eggs were made, and the judges were unable to pick the fillings containing eggs or to pick those not containing eggs. From the writers' viewpoint, the fillings containing no eggs are just as satisfactory and palatable as those containing eggs.

CONCLUSIONS

The growth of *Staphylococcus aureus* was found to be effectively inhibited by fillings made with natural chocolate and natural cocoa according to the formula in this paper.

The inhibitory action was assumed to be due, at least partially, to the pH of the filling. However, the main inhibitory action appeared to be due to a substance or substances contained in the non-fat part of the chocolate or cocoa.

SUMMARY

Natural chocolate (four ounces) and natural cocoa (three ounces) fillings made by the formula as given in this paper have a definite inhibiting action on the rate of growth of *Staphylococcus aureus*. It seems, therefore, that fillings made with chocolate and cocoa as specified should offer little hazard from the standpoint of food illness caused by *Staphylococcus aureus*. The data indicate that natural chocolate and natural cocoa fillings, made by the formula as outlined, without any eggs, are safe. These latter fillings on incubation for 24 hours showed little, if any, growth of staphylococci.

The pH of the fillings and a substance or combination of substances in chocolate and cocoa are responsible for this inhibiting action. The data indicate that this substance or these substances are probably located in the non-fat part of the chocolate and cocoa.

If the food manufacturer makes a natural chocolate or natural cocoa filling with four ounces of chocolate, or three ounces of cocoa, with or without eggs, according to the formula as given, there is little danger of food illness owing to staphylococci.

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STAPHYLOCOCCI AND SALMONELLA CONTROL IN FOODS
IV. EFFECT OF COOKING BAKERY CUSTARDS

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Since there apparently is some doubt in the minds of bakers and health authorities as to the sterility of a properly cooked custard as it is removed from the cooker, it was decided to determine the length of time necessary to hold a custard at the second boil to insure sterility. Custard-filled pies were also used to determine whether or not these custard products would be sterilized by proper baking.

Dack, Woolpert, Noble, and Halliday (1931) have reported that staphylococci inoculated into a cream filler for cakes were destroyed during the cooking of the filler. They also found that staphylococci introduced into various cake batters were killed during the baking process.

EXPERIMENTAL PROCEDURE

Bulk Custard: A standard custard of the following formula was prepared:

Water—1 qt.	Cornstarch—2½ oz.
Sugar—8 oz.	Eggs—2
Salt—⅛ oz.	Dried whole milk—4 oz.

A mixture of three-quarters of the water, one-half the sugar, and the milk powder was brought to a boil. The remainder of the sugar was dry mixed with the starch, the eggs and salt added, and the mixture made into a paste which was thinned by the remainder of the water. This was then added to the boiling mixture and the batch was again brought to a boil where it was held for the desired length of time.

Pies: Baked custard pies were made by filling a raw shell (unbaked pie crust) with a mixture of the following formula:

Water—1 qt.	Cornstarch—¾ oz.
Sugar—8 oz.	Eggs—3
Salt—⅛ oz.	Dried whole milk—4 oz.

The pies were then sprinkled with nutmeg and baked for 25 to 35 minutes in an oven at 246°C.(475°F.).

Bacteriological Procedure: The same strains of *Staphylococcus aureus* and *Salmonella enteritidis* as previously described by Cathcart, Ryberg, and Merz (1941), were used as test organisms in this work. They were counted on blood agar and Endos agar plates, respectively.

Cultures were prepared by diluting inoculated and incubated tubes of nutrient broth. Custards were inoculated in the following manner:

1. In bulk custards varying amounts of the prepared culture of known bacterial content were added to the "thickening" mix. The "length of second boil" was taken as the time elapsing from the start of the second boil (after addition of the thickening mix) to the removal of the kettle from the fire.

2. In baked custard pies varying amounts of the prepared culture of known bacterial content were added to the mixture before filling the raw shells. This gave a known concentration of organisms in the pie as it went to the oven.

TABLE 1

Effect of "Length of Boil" of Custards on Survival of Staphylococcus aureus and Salmonella enteritidis

Series	Length of second boil	Type of cooker	Number of organisms inoculated per gram of custard		Number of organisms per gram of custard after boil	
			Staphylococcus aureus	*Salmonella enteritidis*	*Staphylococcus aureus*	*Salmonella enteritidis*
	min.					
	To boil	Open gas fire	7,700	.....	0	....
1 [1]	2	Open gas fire	7,700	.....	0	....
	5	Open gas fire	7,700	.....	0	....
	2	Open gas fire	15,400	.....	0	....
2 [1]	2	Open gas fire	23,000	.....	0	....
	2	Open gas fire	38,500	.....	0	....
	To boil	Steam	7	.....	0	....
3 [1]	2	Steam	7	.....	0	....
	5	Steam	7	.....	0	....
	To boil	Steam	14	.....	0	....
4 [1]	2	Steam	14	.....	0	....
	2	Steam	35	.....	0	....
	2 (lumpy)	Open gas fire	100	.....	0	....
5 [2]	2	Open gas fire	100	.....	0	....
	2 (lumpy)	Steam	100	.....	0	....
	2	Steam	100	.....	0	....
	To boil	Open gas fire	.....	163,000	....	0
6 [1]	2	Open gas fire	.....	163,000	....	0
	5	Open gas fire	.....	163,000	....	0
	2	Open gas fire	.....	326,000	....	0
7 [1]	2	Open gas fire	.....	488,000	....	0
	2	Open gas fire	.....	815,000	....	0
	To boil	Steam	.....	6,270	....	0
8 [2]	2	Steam	.....	6,270	....	0
	5	Steam	.....	6,270	....	0
	2	Steam	.....	12,540	....	0
9 [2]	2	Steam	.....	18,800	....	0
	2	Steam	.....	31,300	....	0
	2	Steam	.....	1,920	....	0
10 [2]	2 (lumpy)	Steam	.....	1,920	....	0
	5	Steam	.....	1,920	....	0
	5 (lumpy)	Steam	.....	1,920	....	0

[1] Cooked in one-quart batches (one quart of milk). [2] Cooked in two-quart batches (two quarts of milk).

Samples were made up as soon as the custards were cool enough to handle, and dilutions were taken as previously described by Ryberg and Catheart (1941).

Throughout the experiments aseptic precautions were taken as far as conditions would permit. Commercial conditions were attempted, however.

Blood-agar plates were counted at both 24- and 48-hour incubation periods. The Endos agar plates were given 48 hours' incubation before counting.

RESULTS AND DISCUSSION

It will be noted that neither of the organisms survived the cooking process (Table 1). Just bringing the custard to the second boil was sufficient to kill all of the staphylococci and Salmonella inoculated into the mixture. Whether the custards were cooked in a steam kettle or over an open gas fire made no difference. Some of the custards were intentionally made lumpy; however, no organisms survived these either. Also, whether

TABLE 2

Survival of Staphylococcus aureus and Salmonella enteritidis in Commercially Baked Pies

Series	Type of bakery goods	Number of organisms inoculated per gram of filling		Number of organisms per gram of filling after preparation and cooking	
		Staphylococcus aureus	*Salmonella enteritidis*	*Staphylococcus aureus*	*Salmonella enteritidis*
1	Baked custard pie	82	.....	0	....
		123	.....	0	....
		133	.....	0	....
		196	.....	0	....
		205	.....	0	....
		416	.....	0	....
2	Baked custard pie	.....	14	....	0
		.....	21	....	0
		.....	36	....	0
		.....	123	....	0
		.....	185	....	0
		.....	308	....	0

the custards were made in one-quart batches or in two-quart batches made no difference.

The data show that the commercial procedure for custard pies is sufficient to kill both staphylococci and Salmonella organisms (Table 2).

This work indicates that no *Staphylococcus aureus* or *Salmonella enteritidis,* the two organisms chiefly involved in food-illness outbreaks, survive the temperatures of proper baking practice in the preparation of custards. Thus, it is to be expected that properly prepared custards in normal practice will be essentially sterile in relation to *Salmonella enteritidis* and *Staphylococcus aureus* as they leave the cooking kettle or the baking oven, as in the case of baked custard pies.

SUMMARY

Merely bringing custards to a second boil, after the addition of the thickening mix, rendered them sterile of both *Staphylococcus aureus* and *Salmonella enteritidis* with which they had been inoculated.

Baked custard pies, which had been inoculated with *Salmonella enteritidis* and *Staphylococcus aureus* before baking, were sterile in relation to these two organisms as they left the oven.

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ACTION OF VISKING SAUSAGE-CASING MATERIAL ON BACTERIAL GROWTH

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About a year ago I had occasion to investigate an outbreak of carbon-monoxide poisoning which was, in my opinion, erroneously attributed to the eating of cervelat summer sausages, Hall (1939). This investigation led to a study of the remarkable resistance of these sausages to spoilage; they will not spoil either at room temperature or at 37°C.(98.6°F.) in many weeks. *Bacillus botulinus* fails to grow and produce its toxin in them even when considerable numbers of viable spores are inoculated in several places and the sausages are incubated at 37°C. for as long as eight weeks. They spoil within a few hours, however, if placed in water at 37°C. and if inoculated with *B. botulinus* and placed in water at 37°C., strong toxin is produced within a few days, Hall (1940).

Summer sausages are covered with a patented casing called "Visking" which resembles cellophane in appearance. This material protects the sausages against contamination but not against evaporation. Although cervelat summer sausages apparently keep just as well when the casings are stripped off, the question was raised whether the casings play any important role in excluding air, thus favoring the growth of anaerobic bacteria, such as *B. botulinus*. Knowing that the casings are pervious to moisture, the writer believed that they were also pervious to air and that anaerobic bacteria would not grow in the sausages immediately under the casing material. It was decided to test this hypothesis in plates of peptone agar protected with squares of Visking sausage casing in comparison with thin glass covers in a series of experiments similar to those of Sanfelice (1893) and Braatz (1895).

Robert Koch is said to have been the first to cultivate anaerobic bacteria on slabs of agar medium covered with thin pieces of sterile mica, but I have been unable to find an accurate reference to Koch's work. Sanfelice in 1893 cultivated anaerobes in agar between pieces of glass, while Braatz in 1895 showed that obligately aerobic bacteria were inhibited by thin slips of broken glass laid on agar plates. Trenkmann (1898) cultivated *Bacillus tetani*, *Bacillus chauvoei*, and the "bacillus of malignant oedema" in gelatin and agar media between watch glasses, while Streng (1903) used nested glass dishes of a special type for several anaerobes. Then Liefmann (1908) showed that methylene-blue remained decolorized in agar plates covered with thin pieces of glass and that *B. tetani*, *B. botulinus*, and other anaerobic bacilli could readily be grown

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in this manner. The essential facts were confirmed by various other workers, including myself, many years ago. See Hall (1929).

I found that while very small cover-glasses, even one centimeter in diameter, would inhibit the growth of obligately aerobic bacteria, such as *Bacillus subtilis* and other "hay bacilli," much larger covers were necessary to provide suitable conditions for the growth of the obligate anaerobes, because, while a considerable quantity of oxygen is necessary for the growth of the obligate aerobes, a minute amount of oxygen seeping in under a small cover will inhibit the growth of the anaerobes. Consequently, even with a large cover slip, 5 x 4.5 cm., the growth of obligately anaerobic bacteria is usually restricted to a small area in the center, while the inhibition of obligate aerobes extends almost to the very edge.

EXPERIMENTAL PROCEDURE

The action of Visking sausage-casing material was compared with that of glass cover-slips on several different kinds of bacteria. The casing material was cut to the same size as the glass cover, i.e., 5 x 4.5 cm., and in order to identify it in photographs a small portion of the printing was included in each piece. These pieces of Visking were sterilized in beakers of water by autoclave. The glass covers were sterilized in Petri dishes in the dry-air sterilizer. Both were removed by means of flamed forceps and laid side by side upon poured plates of inoculated two per cent agar medium in large Petri dishes, 15 cm. in diameter. Each plate required 20 c.c. of medium.

Certain precautions were found to be necessary. The agar medium had to be completely melted in order to secure a perfectly smooth level surface. It was inoculated only after cooling to about 45°C. (113°F.) and then poured; this avoided excessive moisture. In some cases the sterile agar medium was poured into the plates, allowed to harden, flooded with one c.c. of a fresh culture in glucose broth, and dried before the cover-slips and Visking casing material were applied, but the anaerobes did not grow well in these experiments. The casing material always had to be blotted free of excessive water between sterile filter papers and both the casings and the glass had to be laid carefully on the agar to avoid bubbles of air, although if trapped they could sometimes be pressed out. Then with the aerobes the plates had to be carefully dried to eliminate all moisture from the exposed surface of the casings which otherwise encourage the growth of aerobes on top and obscure the desired result; in the case of the anaerobes this precaution was not so necessary. Finally the humidity of the incubator had to be carefully controlled.

RESULTS

It is well known that bacteria can be divided into four main categories by their relation to air: (1) the strict or obligate anaerobes, (2) the facultative aerobes or microaerophiles, (3) the facultative anaerobes, and (4) the strict or obligate aerobes.

Among the strict anaerobes *B. botulinus*, Types A and B, two strains each; *Bacillus perfringens*, Type A; *Bacillus bifermentans*, two strains, and *Bacillus sporogenes*, two strains; and *Bacillus paraputrificus* were

tested. All behaved essentially alike, failing to grow either in the exposed portions of the plates or under the Visking casings, and growing abundantly in restricted areas from two to four centimeters in diameter with considerable gas production under the glass covers. The growth of *B. botulinus* is illustrated (Plate 1).

Among the facultative aerobes or microaerophiles, *Bacillus tertius*, *Bacillus carnis*, *Bacillus polymyxa*, and *Bacillus histolyticus* were tested.

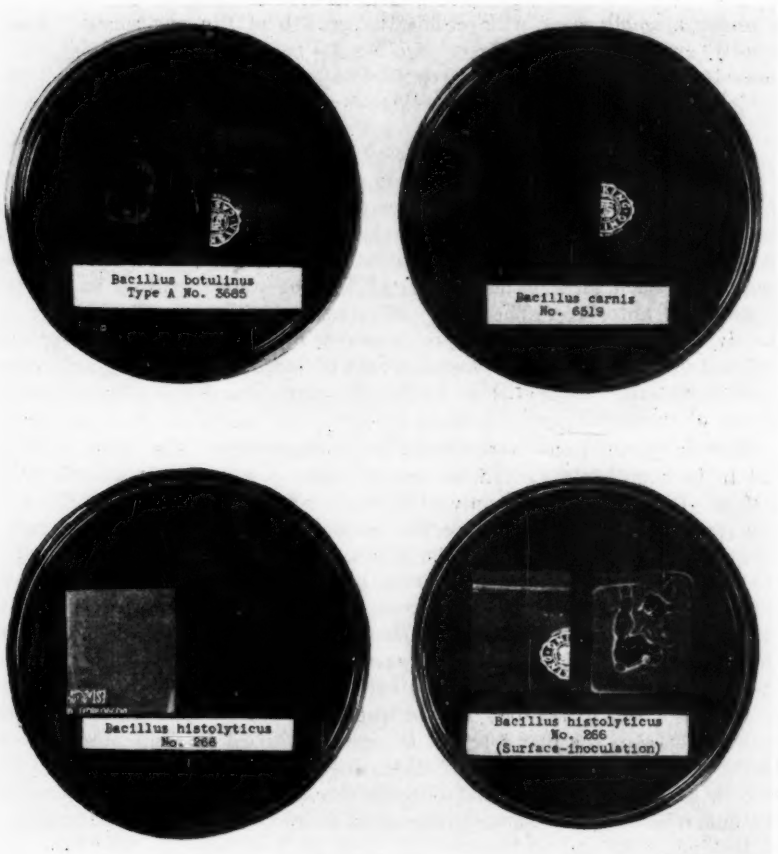

PLATE 1. Action of Visking sausage-casing material and glass covers upon growth of *B. botulinus*, *B. carnis*, and *B. histolyticus* in meat infusion-peptone agar plates.

All produced typical minute colonies in the exposed portions of the plates and under the Visking casing material without visible gas production. The colonies in the exposed portions of the plates were so small, except in the case of *B. polymyxa*, that it was necessary to use a hand-lens or a Beebe-loupe, Hall (1931), to see them and they were poorly visualized by the camera. The absence of visible gas bubbles under the Visking casing may

be attributed to diffusion through this permeable material. All produced colonies and gas bubbles under the glass covers. The growth of *B. carnis* and *B. histolyticus* is illustrated (Plate 1).

B. histolyticus gave a result which was at once peculiar and striking. Minute, almost invisible, colonies developed deep in the agar throughout the entire plate but a heavy growth appeared under the glass and under the casing material which spread to the area between the two and on

PLATE 2. Action of Visking sausage-casing material and glass covers upon growth of *Bact. mucosum, M. aureus, B. anthracis,* and *B. subtilis* in meat infusion-peptone agar plates.

one side nearly two centimeters away from the glass cover. The first time this was seen it was suspected to be accidental or a contamination, but a similar result was obtained in repeated tests and there was no checkable evidence of contamination. This heavy growth was down next to the bottom of the dish not on the surface; in fact no colonies appeared on the surface at all. This is one of the peculiarities of *B. histolyticus*, that

when it is inoculated into melted agar which is then allowed to harden, it acts exactly like an obligate anaerobe. This probably accounts for the fact that it was originally described as such and so regarded for several years until discovery was made that it would grow aerobically, delicately it is true, but in serial transfers as long as one has the patience to transfer it. See Hall (1923). This puzzling behavior may now be explained on the hypothesis that *B. histolyticus* has a marked negative chemotaxis for the air which quickly drives it into the depths of melted agar, yet if inoculated upon the surface, it grows very well. When an agar plate was poured, allowed to harden, flooded with a fresh culture of *B. histolyticus*, dried

TABLE 1

Action of Visking Sausage-Casing Material and Glass Cover-Slips on Various Kinds of Bacteria

Bacteria	Exposed portion	Visking	Glass
Obligate anaerobes *B. botulinus*, Types A and B; *B. perfringens*, Type A; *B. bifermentans, B. sporogenes; B. paraputrificus*	—	—	⊕⊕⊕
Facultative aerobes (Microaerophiles) *B. carnis, B. tertius, B. polymyxa, B. histolyticus*	++	+	⊕
Facultative anaerobes *Bact. coli, Bact. aerogenes, Bact. proteus, Bact. mucosum, Bact. paratyphosum alpha*	+++	+	⊕
Obligate aerobes Group 1—Intermediate *B. anthracis, B. subtilis* (Ford), *M. aureus, B. subtilis* (Cohn)	+++	+	—
Group 2—Strict *B. subtilis* (Marburg), *B. atterimus, Bact. pyocyaneum, Bact. aertryke. Bact. cloacae, Bact. fluorescens*	+++	—	—

and overlaid with glass and Visking covers, colonies appeared all over the exposed surface and under the Visking, but a particularly heavy growth with gas appeared under the glass cover, yet there was no spreading of this heavy growth between the covers as when the agar was inoculated before pouring. *B. histolyticus* undoubtedly leans strongly toward the obligate anaerobes.

B. polymyxa, on the other hand, leans toward the facultative anaerobes as shown by its macroscopic colonies in the exposed portion of the plate and by its poor growth under glass.

The facultative anaerobes tried were *Bacterium coli, Bacterium aerogenes, Bacterium paratyphosum alpha, Bacterium proteus*, and *Bacterium mucosum*. All grew abundantly in the free portions of the plates and under the Visking casings. It was particularly difficult to prevent the growth of *Bact. proteus* on top of the casing also. All grew in a somewhat restricted manner under the glass covers, producing gas. No free gas appeared under the Visking casings. The growth of *Bact. mucosum* is illustrated (Plate 2).

Of the obligate aerobes, *Bacillus anthracis, Bacillus subtilis,* three strains, *Bacillus atterimus, Bacterium pyocyaneum, Bacterium aertrycke, Bacterium cloacae,* and *Micrococcus aureus* were tested. All of these grew freely in the exposed portions of the plates and none grew under glass. The growth of *M. aureus* is illustrated (Plate 2).

B. anthracis, B. subtilis (Ford), *B. subtilis* (Cohn), and *M. aureus* grew in a restricted manner under the Visking covers and may therefore be regarded as leaning toward the facultative anaerobes, while *B. subtilis* (Marburg), *B. atterimus, Bact. pyocyaneum, Bact. aertrycke,* and *Bact. cloacae* failed to grow under Visking and may accordingly be regarded as strict aerobes. The growth of *B. anthracis* and *B. subtilis* (Marburg) is illustrated (Plate 2). The results of these experiments are summarized (Table 1).

CONCLUSIONS

Although the remarkable keeping properties of cervelat summer sausages are due mainly to the prolonged cooking and desiccation to which they are subjected and these keeping properties are not notably altered by stripping off the Visking casings in which they are enclosed, certain observations based upon the above experiments may be made.

(1) The cooking of cervelat summer sausages at 71.1°C.(160°F.) for 12 to 14 hours, or until the insides of certain sausages reach 60°C.(140°F.), doubtless destroys the majority of nonsporulating bacteria, many of which belong to the group of facultative anaerobes. These organisms, some of which might otherwise grow under the Visking casings and cause spoilage, are therefore largely excluded.

(2) The only spore-bearing bacteria which grew under Visking covers were the microaerophiles, and members of the first or intermediate group of obligate aerobes. None of these is likely to cause spoilage and none has even been known to cause food poisoning while all are greatly inhibited by the reduced supply of oxygen under Visking covers.

(3) This leaves finally the obligate anaerobes and strictly obligate aerobes, none of which will grow in agar plates under Visking and, by analogy, almost surely not in sausages. The Visking casings used on cervelat summer sausages not only inhibit the growth of the obligate anaerobes by allowing the passage of oxygen but they also inhibit the growth of the obligate aerobes and to some extent that of many facultative aerobes by restricting the passage of oxygen.

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RELATION OF VOLUME OF DAILY MILK PRODUCTION TO ASCORBIC ACID CONTENT OF COW'S MILK

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Milk has long been recognized as the most efficient and complete food material of the human dietary. Although its chemical composition has been known for many years, its value as a source of vitamins has only recently been given attention. Numerous investigators have reported studies on the ascorbic acid content of cow's milk. Holmes, Tripp, Woelffer, and Satterfield (1939a, 1939b, 1940a, 1940b) have reported studies concerning the ascorbic acid content of certified cow's milk as affected by pasteurization, by breed and seasonal differences, by stages of lactation, and by the age of the cow.

Very few investigators, however, have reported studies concerning the influence of the volume of milk produced by a cow on the ascorbic acid content of her milk. Whitnah and Riddell (1937) reported that there appeared to be no simple relationship between the vitamin C content and yield of milk. Rasmussen, Guerrant, Shaw, Welch, and Bechdel (1936) stated: "With a constant intake of ascorbic acid in the diet of the cow and a decrease in milk production such as usually occurs in the later stages of lactation, one might expect milk of higher ascorbic acid content, especially if the storage capacity of the cow for this substance is limited." In still another investigation Reedman (1937) found that the total output of ascorbic acid varied directly with the quantity of milk produced.

Since there is a lack of agreement in the published investigations and only meager data in the literature consulted, it appeared desirable to accumulate more data in order to clarify the relationship of the volume of milk produced by a cow to its ascorbic acid content. Consequently this investigation of one year's duration was undertaken.

EXPERIMENTAL PROCEDURE

The milk used in this investigation was produced by a herd of mature, high-grade Guernsey and Holstein cows maintained on a certified milk farm in Massachusetts. The ages of the Guernsey cows varied from four to 11 years and of the Holsteins from five to nine years, inclusive. At the start of the experiment the cows were in practically all stages of lactation. They were housed for the one year experimental period in modern dairy barns and were continuously stall fed. At no time during the course of the exper-

iment did they have access to pasture. Since the selected cows were part of a certified herd they were under the direct supervision of an experienced veterinarian and all attendants were under constant medical inspection. Occasionally it was necessary to remove cows from the experiment owing to

TABLE 1

Composition of Dairy and Fitting Rations

Home-Mixed Fitting Ration	
	pct.
Iodized salt	1.0
Calcium carbonate	1.0
Steamed bone meal	1.0
Cane molasses	7.0
Soy bean oil meal (expeller process)	10.0
Yellow corn meal	10.0
Yellow hominy feed	15.0
Wheat bran	25.0
Oats (crimped)	30.0
Dairy Ration	
Calcium carbonate	0.5
Steamed bone meal	1.0
Iodized salt	1.0
Cane molasses	5.0
Soy bean oil meal (expeller process)	5.0
Yellow corn meal	5.0
Cottonseed meal, 41% (hydrolic process 5% fat)	10.0
Corn gluten feed	10.0
Distillers' corn grains, dried	10.0
Yellow hominy feed	10.0
Oats (crimped)	17.5
Wheat bran	25.0
Commercial Fitting Ration	
Carotene	Unknown
Iodized salt	Unknown
Calcium carbonate	Unknown
Molasses	Unknown
Dried beet pulp	Unknown
Alfalfa meal	Unknown
Wheat bran	Unknown
Wheat middlings	Unknown
Soy bean oil meal	Unknown
Crushed oats	Unknown
Corn gluten meal	Unknown
Corn meal	Unknown

a diminished milk flow; however, some of these cows which again freshened before the termination of the study were returned to the experiment.

Consistent feeding conditions were maintained throughout the 12 months' experimental period. The rations of all experimental cows were composed of various combinations of the following ingredients: dairy ration, dairy ration with 6.7 per cent irradiated yeast, home-mixed fitting ration with 6.7 per cent irradiated yeast, a commercial fitting ration with 6.7 per cent irradiated yeast, first and second grades of alfalfa hay, first and second

grades of mixed hay, grass ensilage, and beet pulp. The composition of the dairy and fitting rations are reported (Table 1). The components of the experimental rations were also assayed for their ascorbic acid content by the method of Tillmans as modified by Bessey and King (1933). Five-gram samples were ground with 30 grams of acid-washed sand; 25 to 35 c.c. of an acid mixture containing four per cent metaphosphoric and eight per cent acetic acid were added, as suggested by Musulin and King (1936). The mass was centrifuged, the supernatant liquid was decanted, and the residue was repeatedly extracted until 50 c.c. of extract were obtained. Five cubic centimeters of the extract, diluted to 25 c.c., were titrated with 2,6-dichlorophenolindophenol, as described by Tripp, Satterfield, and Holmes (1937). The results of these analyses are reported (Table 2).

TABLE 2

Ascorbic Acid Content of Components of Experimental Ration

Component	Ascorbic acid per 100 gm. of feed
	mg.
First-grade baled alfalfa	1.883
Commercial fitting ration with irradiated yeast	1.622
Grass ensilage	1.548
Second-grade baled alfalfa	1.380
Good farm hay (one-half alfalfa)	1.334
Second-grade farm hay (all grass)	0.907
Dairy ration with irradiated yeast	0.754
Beet pulp	0.669
Fitting ration with irradiated yeast	0.592
Dairy ration	0.562

Samples from the morning milking (3:30 A.M.) were taken from each of the experimental cows at bimonthly intervals. The samples were immediately transferred to one-ounce flint glass bottles and placed on ice and out of contact with light until they were assayed for their ascorbic acid content. All assays were completed within six to 12 hours after milking. The ascorbic acid content of the samples was determined by the indophenol titration method. Five c.c. of milk were added to a 2.5-c.c. mixture of eight per cent acetic acid and four per cent metaphosphoric acid in a 50-c.c. Erlenmeyer flask. The dye solution was added from a microburette until the final drop produced a faint pink coloration which remained for a 30-second period. The strength of the dye solution was adjusted until it was equivalent to .08 to .10 mg. of ascorbic acid per cubic centimeter and standardized in the manner described by Tripp, Satterfield, and Holmes (1937).

DISCUSSION OF RESULTS

In a previous study Holmes and coworkers (1939b) found that the ascorbic acid content of milk from Guernsey cows was consistently higher than that of Holstein cows. Accordingly the data obtained in this study have been grouped separately for the two breeds. For convenience in correlating the ascorbic acid content of the milk with the daily volume of milk produced, the data for each cow on the day of each assay were tabulated

according to the volume of milk produced. For instance, the data obtained from all cows giving between 20 and 21 liters of milk per day were tabulated in Group I regardless of the ages of the cows and season of the year, since Holmes and coworkers (1939b, 1940b) found in previous studies that the age of the cow and season of the year had little bearing on the ascorbic acid content of milk. The data reported (Table 3) for the Guernsey cows include the number of cows per group, number of assays, variation in the volume of milk produced, variations in ascorbic acid content of the milk, and differences in total daily ascorbic acid output.

TABLE 3

Relationship of Daily Milk Production to Vitamin C Content of Milk and Total Daily Ascorbic Acid Output (Guernseys)

Group No.	Milk per day (liters)	Number of cows	Number of assays	Daily milk production (liters)			Ascorbic acid content of milk (mg. per liter)			Total daily ascorbic acid output (mg.)		
				Min.	Max.	Aver.	Min.	Max.	Aver.	Min.	Max.	Aver.
I	20-21	2	3	20.26	20.91	20.67	16.95	24.02	19.62	355	501	406.0
II	19-20	3	3	19.43	19.55	19.48	16.17	22.99	19.67	314	450	383.3
III	18-19	1	3	18.14	18.71	18.48	16.17	17.93	16.88	300	334	312.3
IV	17-18	3	3	17.16	17.97	17.43	17.96	22.28	20.78	309	400	362.7
V	16-17	2	4	16.20	16.74	16.42	16.56	21.44	19.65	277	347	322.0
VI	15-16	4	6	15.02	15.94	15.47	15.37	20.23	18.88	240	314	291.8
VII	14-15	8	14	14.00	14.88	14.41	16.83	27.00	21.57	236	402	311.1
VIII	13-14	13	26	13.03	13.83	13.46	13.23	28.23	21.65	178	379	291.6
IX	12-13	15	32	12.06	12.99	12.46	16.17	25.48	20.05	156	307	249.7
X	11-12	18	44	11.00	11.94	11.44	15.63	27.40	21.04	165	326	241.0
XI	10-11	19	47	10.00	10.92	10.39	15.22	26.49	20.36	158	278	212.0
XII	9-10	20	47	9.02	9.95	9.48	13.78	25.03	19.67	104	244	186.0
XIII	8- 9	18	58	8.01	8.95	8.46	15.72	29.81	20.51	130	248	173.7
XIV	7- 8	17	32	7.00	7.97	7.56	14.67	31.37	20.56	110	222	155.6
XV	6- 7	11	20	6.39	6.96	6.69	14.95	27.68	20.12	100	183	134.6
XVI	5- 6	4	4	5.15	5.86	5.52	18.35	24.30	19.88	95	142	110.5
XVII	4- 5	2	3	4.01	4.85	4.54	14.39	18.51	16.03	58	90	73.3
XVIII	3- 4	1	2	3.61	3.96	3.79	15.81	16.17	15.99	57	64	60.5

The number of Guernsey cows in the different groups varied from one cow for Groups III and XVIII to 20 cows for Group XII. The number of assays for each group varied from two to 58. The average volume of milk produced by the different groups varied from 3.79 to 20.67 liters per day. It is interesting to note (Table 3) that the smallest average amount of ascorbic acid, 15.99 mg. per liter, was found in the milk of the cow in Group XVIII whose production averaged only 3.79 liters per day. The highest average ascorbic acid value, Group VIII, was 21.65 mg. per liter for 13 cows which produced between 13 and 14 liters of milk per day. However, it should be noted that the smallest amount of ascorbic acid, 13.23 mg. per liter, was also found in a sample of milk obtained from one of the cows of Group VIII. Likewise a further study of the minimum and maximum ascorbic acid values per liter of milk for many of the other samples under consideration showed large variations.

The milk from cows in Groups XIII, XIV, and XV which produced from six to nine liters a day was similar in ascorbic acid content to the milk

of cows of Groups IV, IX, and XI which produced between 17 and 18 liters, between 12 and 13 liters, and between 10 and 11 liters per day, respectively. On the other hand, the milk from two cows of Group I which produced between 20 and 21 liters per day was similar in ascorbic acid content to that of the 20 cows, Group XII, which produced from nine to 10 liters of milk per day.

It is obvious that the total ascorbic acid output per day, which is computed from volume of milk produced and its ascorbic acid content, shows wide variations. The smallest average daily ascorbic acid output, 60.5 mg.,

TABLE 4

Relationship of Daily Milk Production to Vitamin C Content of Milk and Total Daily Ascorbic Acid Output (Holsteins)

Group No.	Milk per day (liters)	Number of cows	Number of assays	Daily milk production (liters)			Ascorbic acid content of milk (mg. per liter)			Total daily ascorbic acid output (mg.)		
				Min.	Max.	Aver.	Min.	Max.	Aver.	Min.	Max.	Aver.
I	21-22	6	6	21.09	21.80	21.44	14.84	19.68	17.38	315	426	372.5
II	20-21	4	9	20.11	20.82	20.38	15.95	22.42	18.63	322	456	380.0
III	19-20	3	3	19.02	19.97	19.34	16.19	18.38	17.59	246	350	306.3
IV	18-19	9	16	18.00	18.95	18.46	12.50	23.18	17.36	237	429	320.3
V	17-18	10	17	17.00	17.96	17.44	11.96	20.56	16.87	205	370	294.4
VI	16-17	11	17	16.11	16.86	16.38	9.93	20.43	16.54	167	340	270.9
VII	15-16	15	26	15.02	15.94	15.45	14.43	20.73	17.76	227	316	274.3
VIII	14-15	13	25	14.00	14.91	14.40	14.39	22.99	18.62	214	326	267.7
IX	13-14	16	27	13.03	13.95	13.49	13.98	22.84	17.91	189	319	241.6
X	12-13	17	35	12.06	12.90	12.51	11.68	22.30	17.46	140	287	218.3
XI	11-12	15	29	11.01	11.98	11.51	10.29	23.36	18.15	119	276	207.6
XII	10-11	15	36	10.04	10.88	10.46	11.88	23.44	17.88	122	253	186.2
XIII	9-10	14	41	9.06	9.95	9.58	13.23	23.25	18.16	126	230	174.0
XIV	8- 9	16	36	8.02	8.98	8.50	9.84	22.42	18.18	85	192	154.4
XV	7- 8	10	15	7.30	7.97	7.70	11.50	20.58	16.92	90	157	130.0
XVI	6- 7	5	7	6.25	6.74	6.48	13.12	20.08	17.97	84	135	116.4
XVII	5- 6	6	6	5.02	5.99	5.69	10.70	20.09	16.44	54	118	94.3
XVIII	4- 5	1	1	4.36	4.36	4.36	25.27	25.27	25.27	110	110	110.0
XIX	3- 4	1	1	3.96	3.96	3.96	19.15	19.15	19.15	76	76	76.0

was obtained for the cow in Group XVIII which produced the smallest amount of milk, between three and four liters per day, while the largest average ascorbic acid output, 406 mg., was found in Group I for cows which produced the largest volume of milk. It will be noticed that there was a definite but not wholly consistent decrease in the average total daily ascorbic acid output as the volume of milk produced decreased with the advance of the lactation period.

The results of the assays obtained from the Holstein cows are reported (Table 4); they were grouped in the same manner as described for the Guernsey cows. The number of cows in each group varied from one cow for Groups XVIII and XIX to 17 cows for Group X.

The number of samples of milk assayed for each group varied from one to 41. The average volume of milk produced in the different groups varied from 3.96 to 21.44 liters per day. The smallest average daily ascorbic acid content of milk was 16.44 mg. per liter for Group XVII for cows producing

between five and six liters of milk. The highest average ascorbic acid was found in the milk of a cow in Group XVIII which produced between four and five liters of milk per day. The milk of Group II, for cows producing 20 to 21 liters of milk daily, contained more ascorbic acid than many of the cows which produced less than 10 liters per day. On the other hand, the milk of cows of Group XVIII which produced between four and five liters per day had a higher ascorbic acid value, 25.27 mg. per liter, than any other group of Holstein cows.

The Holstein cows, like the Guernsey cows, showed wide variations between the minimum and maximum ascorbic acid values obtained for many of the groups. In Group XIV, which produced between eight and nine liters, the minimum ascorbic acid content was 9.84 mg. and the maximum was 22.42 mg. per liter. Also the 11 cows of Group VI, which produced between 16 and 17 liters of milk, showed a difference of from 9.93 to 20.43 mg. of ascorbic acid per liter.

The total daily ascorbic acid output for Holsteins varied from 380 mg. for Group II to 76 mg. of ascorbic acid for Group XIX. As in the case of Guernseys, the smallest average daily ascorbic acid output was for the cow in Group XIX which produced the smallest amount of milk, between three and four liters per day, while the largest daily ascorbic acid output was found in Group II for cows that produced from 20 to 21 liters per day. Thus it is obvious that a very definite, but not wholly consistent, decrease in the total daily ascorbic acid output occurred as the daily volume of milk produced decreased with the advance of the lactation period.

SUMMARY

A one-year investigation was conducted to accumulate data regarding the relation of the daily volume of milk produced to its ascorbic acid content.

Guernsey and Holstein cows from a certified milk herd maintained under controlled feeding conditions were used in this study. The ascorbic acid content of the milk was determined by the indophenol titration method.

The average daily volume of milk produced by the Guernsey cows varied from 3.79 to 20.67 liters. The highest average ascorbic acid value, 21.65 mg. per liter, was found in the milk of the Guernseys producing from 13 to 14 liters daily, while the lowest value, 15.99 mg. per liter, was found in the milk of the cow which produced 3.79 liters per day. The group of Guernseys which produced the largest volume of milk also had the largest ascorbic acid output, 406 mg., during a 24-hour period. The lowest ascorbic acid output was found in the group producing the least amount of milk, between three and four liters per day.

The highest average ascorbic acid value found for the Holsteins was 25.27 mg. per liter in the milk of the cow which produced between four and five liters per day. The group of Holsteins which produced between five and six liters of milk had the lowest average ascorbic acid content, 16.44 mg. per liter. The highest daily ascorbic acid output, 380 mg., was found in the group of cows which produced between 20 and 21 liters of milk per day, while the lowest total ascorbic acid output, 76 mg., was found for the cow which produced but three to four liters of milk per day.

The relation between the volume of milk produced and its ascorbic acid content differed for the two breeds; for instance, the milk from groups of Guernseys which produced less than five liters per day contained the least amount of ascorbic acid, whereas the milk from the Holstein cows which produced less than five liters per day contained the largest amount of ascorbic acid.

The results obtained with both the Guernsey and Holstein cows show that the volume of milk produced per day has little influence on the amount of ascorbic acid found in the milk, that the daily volume of milk produced has a very definite but not wholly consistent influence on the total daily ascorbic acid output, and that as the volume of milk decreases with the advance of the lactation period the total output of ascorbic acid decreases.

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SPICE CONTAMINATION AND ITS CONTROL

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The investigation of spice contamination here reported was undertaken by the Research Laboratory of the National Canners Association as part of a general program to study contamination of ingredients used in canning. The stimulus for this work came out of a report by Jessen, Wood, and Jansen (1934), which focused attention in a very practical manner upon the commercial effect of spice contamination. They found that spore-forming organisms present in a spice mixture containing coriander and white pepper were responsible for swelling in canned chopped hams. Later Jensen (1937) reported that sage and other spice ingredients used in pork sausage were found to contain sufficient bacteria to cause the product to deteriorate rapidly. It was desirable, therefore, to determine whether other spices carried the well-defined spore-forming spoilage types as well as other organisms which might be significant in the spoilage of particular products.

Gibbs (1909) and Tibbles (1912) have suggested the sources of contamination of the crude spice materials, and this topic need not be discussed in the present report. As a general statement it may be said that the treatment and handling of spices at sources of production carry no application of bacteriological control. When the spices are received in this country, therefore, the problem becomes one of removal of contaminating bacteria, particularly where such types may cause spoilage in manufactured products. Emphasis is given here to the industrial phase of the problem. It is appreciated that there may be reaction on aesthetic grounds to the fact that spices may contain large numbers of bacteria. There have been no indications, however, that any defined public-health problem is involved.

A personal visit was made to one spice-manufacturing plant and observations were made of the method of preparing spices with specific reference to means of reducing bacterial numbers. The procedure consisted of the removal of gross foreign material, such as pieces of wood, iron from broken implements, etc., with screens. This was followed by the removal of as much dust and dirt as possible by means of blowers. The spices were then ground and blown through a series of cloth tubes primarily used as screens and not as instruments for removing bacteria. In order to judge the effectiveness of the cleaning treatment at this plant a sample of pepper cleanings and one of the finished product were obtained and tested for their bacterial content. The pepper cleanings had a count of 55,000,000 bacteria per gram while the finished spice had 2,000,000 bacteria per gram. Thus an enormous number of bacteria was removed by the cleaning process used at this plant.

EXTENT AND DEGREE OF SPICE CONTAMINATION

Sources of Samples: A total of 127 samples including 40 different spices and herbs was obtained from three meat packers, four canners, and one spice

manufacturer. Ninety-nine of the samples were of known origin. Most of the samples were in a ground state, but some, for example, bay leaf, were in the "whole form."

Preparation of Samples for Culturing: For whole spices, such as allspice and cloves, a 10-gram sample was used. Where the spices or herbs were of a bulky nature, a two-gram sample was employed, and in the case of ground spices, a one-gram sample was used. The weighed sample of spice or herb was placed in a flask with 100 c.c. of sterile water and shaken vigorously for five minutes in order to suspend the bacteria. Appropriate dilutions of the prepared suspensions were pipetted into various media.

Cultures: For the total bacterial counts of unheated spice suspensions, cultures were made in Difco brom cresol purple (B.C.P.) tryptone dextrose agar. Incubation was at 37°C.(98.6°F.) for 48 hours. For the detection of acid-tolerant bacteria and for molds, wort agar cultures were prepared and incubated at room temperature for five days. After boiling for five minutes the suspensions were cultured for spores in B.C.P. tryptone dextrose agar. These cultures were incubated at 37°C. and 55°C.(131°F.) for 48 hours. For the detection of spores of aerobic gas-producing bacteria, cultures were prepared in the medium of Jensen, *et al.* (1934) and incubated at 37°C. for five days. Many of the heated spice suspensions also were cultured for thermophilic anaerobic bacteria (not producing H_2S) using liver broth medium—Cameron (1936)—and for sulfide-spoilage bacteria (thermophilic anaerobes producing H_2S) using sulfite medium—Cameron (1938). All cultures were incubated at 55°C. for 48 hours. Finally to detect the presence of putrefactive anaerobic spores, cultures were prepared in liver broth medium and incubated at 37°C. for five days. The results of the bacteriological survey of spices are given (Table 1).

In general, the results showed marked variations in the bacterial counts of similar spices. Black pepper was the spice most heavily contaminated. Total counts for seven samples ranged from 1,200,000 to 16,300,000 per gram. Counts for molds, acid-tolerant bacteria, and mesophilic aerobic types were correspondingly high, while counts for thermophilic aerobic spores were relatively low. Putrefactive anaerobes were not encountered in any of the 116 samples examined for these types. Spices like the peppers, coriander, and marjoram varied widely in the counts of the aerobic spore-forming organisms producing gas in Jensen's medium. Cloves, whole or ground, with a maximum count of 12,000 organisms per gram, were the least contaminated of all the important spices tested.

Jensen (1935), James (1938), and Hall (1938) also found high bacterial counts in certain spices, and Jensen concluded that spices were grossly contaminated in the countries where grown and that the American importers did very little in adding to their microbic content.

From the results recorded above it would appear that practical handling of the problem of bacterial contamination of spices depends upon sterilization, since it does not appear likely that untreated spice of low bacterial content can be prepared readily. Complete sterilization is not a usual commercial requirement for spices any more than for starch, flour, or other ingredients, but there is a need for treatment which would result in considerably lower bacterial counts. The wide variation in counts for different

TABLE 1

Bacterial Content Per Gram of Material[1]

Kind of material	Total bacteria, tryptone agar at 37°C. (98.6°F.)	Acid-tolerant bacteria, wort agar at room temperature	Molds, wort agar at room temperature	Total spore formers, tryptone agar at 37°C. (98.6°F.)	Spores—gas formers in Jensen medium at 37°C. (98.6°F.)	Aerobic spore formers, tryptone agar at 55°C. (131°F.)
Ground black pepper	(7) 1,200,00[2] to 16,300,000	(7) 520,000 to 9,400,000	(7) 80,000 to 1,300,000	(7) 97,000 to 580,000	(7) 1,000 to 10,000	(2) 1,200 to 1,800
Ground white pepper	(5) 7,000 to 220,000	(5) 0 to 80,000	(5) 2,700 to 230,000	(5) 0 to 300	(5) 0 to 100	(3) 0 to 500
Decorticated pepper	(4) 1,140,000 to 1,380,000	(4) 460,000 to 1,160,000	(4) 20,000 to 110,000	(4) 73,000 to 470,000	(4) 10,000 to 100,000	.....
Pepper cleanings	(1) 55,000,000	(1) 9,500,000	(1) 300,000	(1) 750,000	(1) 100,000	.....
Whole allspice	(5) 106,000 to 1,000,000	(5) 200 to 780,000	(5) 600 to 70,000	(5) 10 to 200,000	(5) 0 to 1,000	(1) 65
Ground allspice	(3) 64,000 to 1,950,000	(3) 0 to 100,000	(3) 50,000 to 220,000	(3) 100 to 3,000	(3) 0 to 100	(2) 0 to 200
Whole cloves	(5) 190 to 12,000	(5) 0 to 3,700	(5) 10 to 14,000	(5) 0 to 20	(5) 0 to 100	(1) 0
Ground cloves	(3) 8,000 to 12,000	(3) 0	(3) 0 to 2,000	(3) 0	(3) 0	(1) 0
Crushed cinnamon	(3) 8,000 to 90,000	(3) 1,000 to 6,000	(3) 600 to 12,000	(3) 10 to 1,500	(3) 0 to 50	.....
Ground cinnamon	(3) 12,500 to 36,000	(3) 0 to 7,000	(3) 9,000 to 60,000	(3) 0 to 1,100	(3) 0 to 100	(1) 100
Whole coriander	(1) 28,000	(1) 0	(1) 4,500	(1) 100	(1) 100	.....
Ground coriander	(4) 37,000 to 800,000	(4) 0 to 100,000	(4) 1,400 to 240,000	(4) 100 to 450,000	(4) 100 to 100,000	(2) 100 to 200
Whole thyme	(5) 675,000 to 2,700,000	(5) 0 to 445,000	(5) 12,000 to 50,000	(5) 500 to 10,000	(5) 500 to 5,000	.....
Ground thyme	(3) 6,600 to 200,000	(3) 200 to 20,000	(3) 100 to 170,000	(3) 100 to 3,400	(3) 50 to 1,000	(2) 0 to 35
Whole marjoram	(3) 80,000 to 290,000	(3) 0 to 19,000	(3) 6,000 to 7,500	(3) 1,100 to 4,000	(3) 50 to 5,000	.....
Ground marjoram	(2) 39,000 to 820,000	(2) 0 to 40,000	(2) 2,200 to 3,000	(2) 700 to 900	(2) 1,000 to 10,000	(1) 0
Whole mace	(1) 6,400	(1) 12,000	(1) 3,200	(1) 200	(1) 0	.....
Ground mace	(3) 2,800 to 31,000	(3) 0 to 1,000	(3) 400 to 9,000	(3) 0	(3) 0 to 100	(2) 0
Whole E. India nutmeg	(1) 1,875	(1) 0	(1) 13	(1) 0	(1) 0	.....
Ground E. India nutmeg	(1) 1,200	(1) 600	(1) 700	(1) 0	(1) 0	.....
Ground nutmeg	(3) 1,000	(3) 0	(3) 1,600	(3) 100	(3) 0	(2) 0
Whole bay leaves	(4) 15,000 to 63,000	(4) 150 to 9,000	(4) 150 to 6,500	(4) 100 to 10,500	(4) 0 to 5,000	.....

		to 31,000		to 1,000		to 9,000				to 100		
Whole E. India nutmeg	(1)	1,875	(1)	0	(1)	13	(1)	0	(1)	0		.....
Ground E. India nutmeg	(1)	1,200	(1)	600	(1)	700	(1)	0	(1)	0		.....
Ground nutmeg	(3)	1,000	(3)	0	(3)	1,600	(3)	100	(3)	0	(2)	0
Whole bay leaves	(4)	15,000 to 63,000	(4)	150 to 9,000	(4)	150 to 6,500	(4)	100 to 19,500	(4)	0 to 5,000		.....
Ground paprika	(4)	520,000 to 2,400,000	(4)	150,000 to 480,000	(4)	2,000 to 70,000	(4)	0 to 100	(4)	0 100	(1)	0
Ground Spanish paprika		.....		.....		.....		.....		.....	(1)	1
Ground Hungarian paprika	(10)	.....		.....		.....		.....		.....	(10)	0 to 314
Ground ginger	(3)	60,000 to 250,000	(3)	0 to 21,000	(3)	200 to 2,000	(3)	100 to 700	(3)	0 to 1,000	(2)	0 to 100
Sweet basil	(2)	26,000 to 525,000	(2)	750 to 160,000	(2)	50 to 250	(2)	0 to 1,200	(2)	0		.....
Savory	(2)	4,000 to 250,000	(2)	0	(2)	300 to 450	(2)	0 to 400	(2)	0 to 100	(1)	300
Ground red pepper	(2)	2,190,000 12,500,000	(2)	0 to 2,000,000	(2)	500,000 to 1,220,000	(2)	100 to 300	(2)	0		.....
Ground mustard	(2)	1,500 to 40,000	(2)	0	(2)	0 3,000	(2)	0 to 100	(2)	0	(1)	0
Ground celery seed	(2)	230,000 to 1,150,000	(2)	16,000 to 95,000	(2)	3,000 to 10,000	(2)	100 to 260,000	(2)	1,000 to 50,000	(2)	0 to 200
Onion powder	(2)	6,000 to 222,000	(2)	0	(2)	0 to 900	(2)	0 to 100	(2)	0	(2)	0
Ground Mexican chili pepper	(1)	2,300,000	(1)	350,000	(1)	100,000	(1)	1,910	(1)	0		.....
Whole Japan chilies	(1)	2,000	(1)	1,625	(1)	375	(1)	0	(1)	0		.....
Ground chili pepper	(1)	9,000,000	(1)	410,000	(1)	10,000	(1)	800	(1)	0		.....
Rubbed sage	(1)	24,000	(1)	123,000	(1)	0	(1)	0	(1)	0		.....
Ground sage	(1)	270,000	(1)	130,000	(1)	20,000	(1)	100	(1)	100		.....
Whole allepy pepper	(1)	72,000	(1)	27,000	(1)	2,000	(1)	78,000	(1)	0		.....
Ground turmeric	(1)	3,100,000	(1)	5,000	(1)	3,000	(1)	310,000	(1)	0		.....
Parsley seed	(1)	3,000	(1)	350	(1)	150	(1)	150	(1)	50	(1)	0
Ground arogona	(1)	38,000	(1)	6,000	(1)	4,000	(1)	100	(1)	50	(1)	0
Ground caraway	(1)	310,000	(1)	19,000	(1)	5,000	(1)	300	(1)	10,000	(1)	100
Ground cardomon	(1)	44,000	(1)	47,000	(1)	1,000	(1)	0	(1)	100	(1)	0
Ground Cayenne pepper	(1)	3,800,000	(1)	400,000	(1)	200,000	(1)	300	(1)	10,000	(1)	0
Ground sage	(1)	170,000	(1)	2,000	(1)	11,000	(1)	200	(1)	100	(1)	0
Ground cumin	(1)	1,200,000	(1)	600,000	(1)	200,000	(1)	200	(1)	0	(1)	100
Ground curry	(1)	550,000	(1)	0	(1)	4,300	(1)	4,000	(1)	50	(1)	3,000
Ground garlic	(1)	90,000	(1)	0	(1)	200	(1)	0	(1)	100	(1)	0
Ground Hungarian pepper	(1)	650,000	(1)	220,000	(1)	40,000	(1)	0	(1)	0	(1)	0

[1] Non-putrefactive anaerobes were found in two samples of thyme and in one sample each of bay leaves, black pepper, white pepper, cardomon, and celery seed. Fifty-two materials were tested for thermophilic anaerobes (not producing H_2S). These spores were found only in one sample each of arogona and Hungarian pepper. Fifty-two materials were tested for thermophilic anaerobes (producing H_2S). All samples were negative for these spores. [2] Numbers in parentheses indicate the number of samples tested.

samples of similar spices suggests that some measure of control might be applied in maintaining a proper bacterial condition in spices from the time of their preparation until their use. This, however, would depend upon improvement in the established food-handling customs of spice exporting countries and nothing has come to attention that would suggest an attitude that is promising in this respect. On the contrary it would appear that manufacturers in this country must develop and apply their own control measures.

STERILIZATION METHODS

Black pepper was chosen as the principal test spice for study of sterilization methods both because of its extensive use and because of its high contamination. The various treatments that were applied, and their effect, are discussed individually.

Heat: Sterilization by heat would offer a relatively simple solution to the problem of contamination if it could be carried out with little or no reduction in the potency of the spice, and without modification of its flavor. Experiments were therefore conducted to determine the effect of heat on bacterial count and on potency and flavor.

A preliminary series of range-finding experiments was made in which samples of pepper were autoclaved in test tubes, sealed and unsealed. In the first experiment the spice carried contamination of appreciable resistance and there was a limited survival even after a treatment of 20 minutes at 115°C.(239°F.). Specifically the reduction accomplished was from 13,000,000 to 200 bacteria per gram. This reduction was much greater than would be necessary for acceptable commercial quality.

In another test black pepper was spread in a large porcelain pan to a depth of about one-half inch. The pan was covered with brown paper and autoclaved at 15 pounds pressure at 121°C.(250°F.) for 15 minutes. Following this the spice was dried at 35°C.(95°F.) for 20 hours. The reduction in number of bacteria by this treatment was from 83,000,000 to about one per gram. This lot was subjected to a potency test in four laboratories connected with the meat-packing industry. Methods of assay varied somewhat but the preferred method consisted of mixing the test samples with ground beef, cooking the mixture, and finally judging the flavor. The consensus was that the unheated sample was approximately 10 per cent stronger in flavor and odor.

Concluding experiments were made in which spice samples were heated for 5-, 10-, and 15-minute intervals at 5, 10, and 15 pounds of steam pressure. Original counts at 37°C.(98.6°F.) were 26,000,000 vegetative cells and 750,000 spores per gram. The lot receiving the lightest heat treatment, five minutes at five pounds pressure, gave a count of 1,300 vegetative cells and 100 spores per gram. Other lots were practically sterile. Potency tests of spices heated at 15 pounds pressure for 15 minutes indicated flavor destruction amounting to about 10 per cent.

These results show that moist heat is an effective means of sterilizing black pepper. There is evidence that the spice contamination will vary in its resistance, but it appears that a relatively mild treatment is effective in reducing contamination to a low level. The economic disadvantage resulting

from a loss of 10 per cent potency will probably be more than compensated for by spoilage reduction.

Ethylene Oxide: Cotton and Roark (1928) were the first to report the use of ethylene oxide as a fumigant. They stated that insects buried in overstuffed furniture, sealed in packages of cereals, or present in jars of rice were killed with ease. No deleterious effect was noticeable when foodstuffs, such as nut meats and dried fruits, were treated. Cotton and Young (1929) found that by mixing carbon dioxide with ethylene oxide the toxicity was considerably increased and the fire hazard was reduced. Gross and Dixon (1937) patented a method for sterilizing foodstuffs, soil, etc., by means of ethylene oxide used at room temperature. It was also found to be effective in destroying pure cultures of various types of bacteria, among which were anaerobic types such as *Clostridium botulinum.* Griffith and

TABLE 2

Effect of Ethylene Oxide on Bacterial Content of Spices

No.	Material	Counts per gram of spice	
		Total (vegetable cells and spores)	Spores
1	Top ¼ inch layer of pepper	380	Less than 20
2	Bottom ¼ inch layer of pepper	1,480	Less than 20
	Control (pepper)	16,000,000	48,000
3	Top ¼ inch layer of cinnamon	20	Less than 20
4	Bottom ¼ inch layer of cinnamon	50	Less than 20
	Control (cinnamon)	36,400	10,000

Hall (1938) patented a method for sterilizing dry food materials by using ethylene oxide at an elevated temperature and *in vacuo.*

The effect of ethylene oxide treatment on the bacteria present in spice was studied in several ways. In a preliminary series of experiments five-gram lots of black pepper were weighed into 300 c.c. Erlenmeyer flasks. In some tests ethylene oxide (.7 c.c. was applied in direct contact with the spice. In others the agent was dropped into a small tube suspended within the flask above the spice. Some of the flasks had been evacuated prior to the addition of the agent. Flasks were exposed for 24 hours at room temperature, at 37°C.(98.6°F.), and at 50°C.(122°F.). The total bacterial counts and spore counts indicated that the agent was most effective under vacuum in direct contact with the spice and at the higher temperature of exposure; for example, before treatment one pepper sample had a total count of 16,000,000 and a spore count of 48,000 per gram. After exposure of the spice away from the agent *in vacuo* at 50°C. for 24 hours, the total count was reduced to 480 per gram. With the agent directly in contact with the spice *in vacuo,* the total count was reduced to 90 per gram. The spore count in each case was less than 20 per gram of spice.

Upon the suggestion of W. S. Hilpert of the Miner Laboratories, Chicago, the following series of tests was carried out. Black pepper and cinnamon in 100-gram lots were placed in pint Mason jars with the covers loosely fitted, and exposed to ethylene oxide in a three-gallon aluminum vacuum kettle. The kettle was placed in a water bath at a temperature of approxi-

mately 85°C.(185°F.) and evacuated to 27 inches. Then 15 c.c. of ethylene oxide were admitted. This resulted in a reduction of the vacuum to 23 inches. The kettle was kept at approximately 80°C.(176°F.) for five hours during which time the vacuum dropped to 18 inches. After this time the kettle was aerated with filtered air for 20 minutes.

In order to determine whether the ethylene oxide had reached the spices in the bottom of the jars, a five-gram sample of each spice from the top layer and the bottom layer was used for bacterial counts. The results of cultures incubated at 37°C.(98.6°F.) for 48 hours (Table 2) indicate that after the ethylene oxide treatment the spices approached sterility even at the bottom of the jars.

Tests were made for residual oxide by using freshly treated black pepper and some black pepper which had been sterilized by heat. Both pepper samples were inoculated with aerobic spores and then were stored overnight in tightly stoppered tubes. Tests made on suspensions of each sample gave approximately the same counts, indicating that there was no residual ethylene oxide in the freshly treated spice.

A series of tests was made to determine the difference in degree of the destructive action of ethylene oxide upon aerobic spores which had been dried on sand, a nonabsorbing material, and on spores which had been dried in starch, an absorbing material. The starch used in this series of tests was considered analogous to finely ground spice. The samples of sand and starch were placed in loosely capped jars and were treated with 15 c.c. of ethylene oxide in a dry condition as in the previous experiment. The vacuum ranged from 28 inches at the start to approximately 14 inches after five hours, and the temperature of the water bath varied from 80° to 86°C.(176 to 186.8°F.). Total counts of the materials after the treatment showed 94.9 per cent reduction on the sand and 99.6 per cent reduction in the starch.

Another test was made in which the sand and starch containing the aerobic spores were treated with ethylene oxide in a vacuum kettle in which was placed a beaker of water to supply moisture. The total counts made after the treatment showed the materials to be sterile. It was concluded from these tests that ethylene oxide was more effective when used in a moist condition than in a dry condition. Spices would carry enough moisture to satisfy such a condition.

It was considered desirable to compare the effectiveness of ethylene oxide with ethylene glycol which, according to Cotton and Roark (1928), is formed when ethylene oxide is heated in the presence of water. Flasks were set up according to the following protocol, which gives also the percentage reduction of spores.

No.	Treatment	Per cent spore reduction
1	1 c.c. ethylene glycol added to 100 c.c. liver broth spore suspension, shaken 30 minutes	26
2	1 c.c. ethylene oxide added to 100 c.c. liver broth spore suspension, shaken 30 minutes	100
3	1 c.c. ethylene oxide added to 100 c.c. liver broth, then boiled 5 minutes, cooled, and inoculated with spore suspension, shaken 30 minutes	22

It is obvious from these results that ethylene oxide is a much more effective bactericidal agent than ethylene glycol.

Formaldehyde: Formaldehyde was used as a matter of incidental investigation because of its recognized germicidal properties; however, its acceptability in commercial practice is not a subject of comment in this paper. Several tests were made in vacuum and at atmospheric pressure with black pepper. Reduction in total count from 16,000,000 to 600 per gram was obtained. To what extent this was due to actual destruction was not established, however, because other experiments indicated that failure of growth in plate cultures may have been due to inhibition induced by the reagent. The bacteriostatic property of formaldehyde is of course not a new observation. Also it was not possible to distinguish between reduction in total count and spore count because the test for spores called for boiling before spore plates were made, and the combined formaldehyde and boiling treatments may have been responsible for the apparent spore destruction.

Miscellaneous: In addition to the methods of treatment described, tests have been made with ultra-violet light, ethyl formate, and ethyl ether, all of which have been ineffective. Ethyl formate was used because, like ethylene oxide, it is used as a fumigant and it was suggested that its destructive powers might extend to bacteria. Ethyl ether was used on the assumption that it might act as a solvent for certain cell constituents, in which case sterilization would result from "induced autolysis." This mechanism was suggested in possible explanation of the results obtained with ethylene oxide, but conditions of treatment that were effective when the latter was used did not apply where the test was made with ether.

SUMMARY

In general, spices as received by importers showed heavy contamination with bacteria. There may be an appreciable reduction in the bacterial content during their processing at the manufacturing plants through mechanical removal by screening methods.

Different lots of black pepper were subjected to heat, ranging from a treatment of five pounds of steam pressure for five minutes to 15 pounds of pressure for 15 minutes. Results indicated that the spice contamination varied in its resistance, but that a relatively mild treatment is effective in most instances. Cooking tests indicated a loss in potency of about 10 per cent following a heat treatment of 15 minutes at 15 pounds of pressure.

Practical sterility of spices was obtained by the use of ethylene oxide. The effectiveness of this agent appeared to be due to a true germicidal property and not to a bacteriostatic action. Ethylene glycol was much less effective than ethylene oxide.

Only moderate destruction of vegetative forms or spores was accomplished by the use of ethyl formate.

Formaldehyde was effective, especially against spores, although destruction may have been due to the combined effect of residual formaldehyde and the heat treatment used to destroy vegetative cells. With vegetative cells there may be a combined effect of destruction and bacteriostasis to account for the reductions which were noted.

Ultra-violet light and ethyl ether were ineffective in the tests which were made.

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ASCORBIC ACID CONTENT OF FOUR VARIETIES OF RAW WINES[1]

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An inspection of the literature relative to the ascorbic acid content of wines reveals the lack of work done on American wines. Seemingly, investigators in this country have confined their studies largely to the vitamin content of fruits or juices pressed from those fruits, although Morgan *et al.* (1939) report from preliminary experiments that wines contain little ascorbic acid. Continental investigators have published data on various vitamin determinations on wines as well as fresh juices. Randoin (1928, 1936), working first with grape juice, found a small amount of ascorbic acid in freshly pressed juice. With white wine she mentions the presence of very little ascorbic acid—much less than is found in the juice of fresh grapes. This amount rapidly disappears when the wine is concentrated. Merjanian (1926) found that new wines contain ascorbic acid and that old wines contain only traces of it. Minz and Serianni (1934), reviewing the work of other investigators, report only a small amount of the vitamin in wine. In their work on apples, Fellers, Cleveland, and Clague (1933) found that benzoated or pasteurized ciders over 40 hours old failed to retain an appreciable amount of ascorbic acid.

In connection with the work done on the ascorbic acid content of several varieties of grapes by Yarbrough and Satterfield (1938), it was thought wise to determine the ascorbic acid content of certain wines. Accordingly, samples of four varieties of wines were obtained: apple *(Pyrus Malus)*, peach *(Prunus persica)*, white and black scuppernong *(Vitis rotundifolia)*. All of these wines were in the stage just prior to the finishing process; that is, the final amount of sugar had not been added to make the wine as yet fit for drinking purposes. With this exception, the wines were identical with those on the market.

EXPERIMENTAL PROCEDURE

The method used was essentially the oxidase method of Tauber and Kleiner (1935) and Tauber, Kleiner, and Mishkind (1935). The mercuric acetate method of Emmerie (1934) and Emmerie and Van Eekelen (1934)

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was not found to be practical because it was not possible to rid the solution entirely of the hydrogen sulphide. Nor was it possible to determine the vitamin simply by titration with a standard indicator, for the sodium bisulphite added at the winery to prevent decay of the grapes before they can be made ready for fermentation reacted to a marked degree with the dye. The method, modified for wine, is as follows:

To 50 c.c. of wine in a 100-c.c. volumetric flask were added five c.c. of M acetate buffer of pH 6, and the solution was diluted to the mark with the oxidase solution. The oxidase solution was prepared by mincing 250 grams of either Hubbard or summer squash in a meat chopper and then shaking with 750 c.c. of 30 per cent ethyl alcohol to extract the enzyme, and finally filtering. The enzyme solution, which is specific for ascorbic acid, was buffered to pH 5-6 and tested to determine if 10 c.c. would oxidize .5 mg. of pure vitamin C in 30 minutes. This solution keeps well in a refrigerator. The wine with the oxidase was then placed in an oven and held at a temperature of 38°C.(100.4°F.) for 30 to 60 minutes.

While this was incubating, another portion (50 c.c.) of the same wine was diluted to 100 c.c. with five per cent acetic acid, and an aliquot was titrated with a standard solution of 2,6-dichlorobenzenoneindophenol to determine the total reducing power of the wine. The standard indicator was made by dissolving 125 mg. of the indophenol in hot distilled water and diluting to 50 c.c. Ten c.c. of this were diluted to 100 c.c., and the resulting dye was standardized against pure ascorbic acid.

After the incubation period for the wine was finished, the wine was removed from the oven, one c.c. of two per cent sulphuric acid was added to stop enzyme activity in each aliquot, and an aliquot was titrated as described above. This second reading should be lower than the first, the difference being due to the reducing power of the now oxidized ascorbic acid. As a check on the degree of activity of the enzyme, a measured amount of ascorbic acid was added to some of the wine, the resulting solution was treated with the oxidase as above, and then incubated. The oxidase destroyed the pure vitamin.

The readings from the various wines did not show enough difference to justify the assumption that there is any ascorbic acid present in the wines tested; that is, the differences in the titration values were so small as to be considered experimental error, or there were no differences at all.

DISCUSSION

This is not surprising, however, when one considers the fact that the vitamin is very susceptible to oxidation, that the process of fermentation presents ample opportunity for the destruction of the vitamin, and that the wines had been stored for a year or slightly less.

Other workers have met with similar results. Büsig and Raabe (1938) have found that grape juice preserved with sulphurous acid is void of ascorbic acid. Merjanian (1926), as cited before, reports that old wines contain only traces of ascorbic acid; and Randoin (1928, 1936) reports that only fresh grape juice contains an appreciable amount of ascorbic acid, indicating the gradual loss of the vitamin with storage of the juices or wines.

SUMMARY

A study was made of the ascorbic acid content of apple, peach, and black and white scuppernong wines that had been stored for a year or less. No ascorbic acid was found in the wines.

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DETERMINATION OF IRON IN THREE DIETS

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Careful analyses for the iron content of three diets have brought out some new points having a general bearing on the precise determination of this element in food materials. Determinations were made by the ferron, mercaptoacetic acid, and *aa'*-dipyridyl methods.

Diet 1 was essentially that described by Chaney and Ahlborn (1939). Diet 2 was the minimum-cost, adequate diet of Stiebeling and Ward (1933). Diet 3, a new low-cost diet being investigated here, was made up as follows, in pounds on a weekly basis: corn meal, 5; salt pork, .50; oleomargarine, 1; sweet potatoes, 3; sorghum molasses, .63; sugar, .50; black-eyed peas, 1; evaporated milk, 1.88; turnip greens, 1; turnips, 1; onions, 1; canned tomatoes, .60; white flour, 1; and added vitamins.

REAGENTS

Fifteen N nitric acid, 9 N perchloric acid, 17 N acetic acid, 36 N sulfuric acid, 6 N hydrochloric acid, .6 N trichloroacetic acid, and 4 N aqua ammonia were prepared free of iron by distillation methods. Ten N iron-free sodium hydroxide was obtained by electrolysis in platinum at five amperes for 30 hours, with a rotating cathode. The 30 per cent hydrogen peroxide used was shown to be practically free of iron by blank tests. Sodium thiosulfate was purified as described by Hill (1930). Potassium dihydrogen phosphate and sodium acetate trihydrate were crystallized twice with centrifugal draining, and dried by appropriate vacuum desiccation.

A buffer solution of pH 2.6 for the ferron tests was prepared by making 10.21 grams of recrystallized potassium acid phthalate and 5.95 grams of 20.24 per cent (constant boiling) hydrochloric acid to a liter with distilled water. The buffer solution of pH 3.6 for the dipyridyl tests contained 30 ml. of 17 N glacial acetic acid and 5.1 grams of sodium acetate trihydrate per liter. The ferron and dipyridyl reagents were .2 per cent aqueous solutions of the Eastman and G. F. Smith products, respectively. Standard ferric iron solutions were made up as described by Yoe and Hall (1937); it was found that permanganate cannot be substituted for bromine in preparing these. The mercaptoacetic acid reagent was made as described by Leavell and Ellis (1934).

The sodium hydroxide was kept in ceresin, the other reagents in Pyrex bottles.

ASHING OF SAMPLES

Wet Ashing: Dried, uniform, 10-gram samples were ashed by the perchloric acid method of Gerritz (1935) by successive treatment with a 60-ml. and one or two 30-ml. portions of 15 N nitric acid and 12 to 24 ml. of 9 N perchloric acid. This procedure calls for a digestion time of about 1.5 hours, but in view of observations noted in the next paragraph, the time

used in the first set of digests was three to five hours. Digests of the duplicate and some triplicate samples covered 12 to 18 hours to insure maximum ashing. The solutions were eventually evaporated to between 20 and 50 ml., cooled, diluted, then filtered promptly through a platinum Munroe or Pyrex filtering crucible to remove silica. The filtrate was boiled for an hour to hydrolyze pyrophosphate, then cooled, and made to 250 ml.; 50-ml. aliquots were used in the iron analyses.

Regardless of digestion time the solutions finally obtained were usually yellow to yellow-green in color. In two cases where the initial evaporation was continued to a very low volume the solutions were colorless, but this result could not be repeated consistently without risk of losing phosphorus. Evaporation of aliquots to dryness gave small dark deposits, probably carbonaceous, and sometimes containing yellow and green solids.

Dry Ashing: Other 10-gram samples were ashed to colorless products in deep fused silica beakers, at temperatures raised gradually to 500°C. (932°F.), in an electric furnace.

METHODS OF ANALYSIS

Seven groups of analyses were made for each diet: (1) A 50-ml. aliquot was evaporated in silica to about 20 ml., cooled, and analyzed by the ferron method described below. (2) A 50-ml. aliquot was evaporated to dryness and bleached by successive treatment—repeated when necessary—with .5-ml. to 1-ml. portions of 36 N sulfuric acid, 30 per cent hydrogen peroxide, and 9 N perchloric acid, with intermediate and final evaporation to dryness at temperatures not exceeding 230°C.(446°F.). The residue was taken up with 1 ml. of 6 N hydrochloric acid followed by 20 ml. of water, and analyzed promptly by the ferron method. (3) A 50-ml. aliquot was treated exactly as in 2, but after the residue was dissolved the solution was heated overnight at 80°C.(176°F.) under reflux to hydrolyze pyrophosphate, before the analysis was continued. (4) A 50-ml. aliquot was evaporated to dryness in a silica beaker and the residue was heated electrically at gradually increasing temperatures until colorless. The analysis was then completed as in 3. (5) A 50-ml. aliquot was treated as in 2, and the bleached residue was treated with .5 ml. each of 18 N sulfuric acid and 30 per cent hydrogen peroxide, and taken up with 20 to 40 ml. of water. After boiling for 30 minutes to hydrolyze pyrophosphate, the solution, or a suitable aliquot, was analyzed by the mercaptoacetic acid method described below. (6) The residue from an electrically ashed 10-gram sample was taken up with 3 ml. of 6 N hydrochloric acid followed by water. The solution was heated under reflux overnight, then analyzed by the ferron method. (7) A five-gram sample was analyzed for ionizable iron without ashing, by a spectrophotometric method based on the colorimetric methods of Borgen and Elvehjem (1937), which proved unsatisfactory with colored or colloidal solutions.

Analyses were made with a Coleman 10-S-30 spectrophotometer, with water or suitable blanks as reference liquids.

Ferron Method: The acid solution, in volume about 20 ml., was treated with a .1-ml. to 1-ml. portion of 30 per cent hydrogen peroxide, and the excess was removed shortly afterward by boiling. A tiny piece of Congo red paper was added to the cooled solution, then 10 N sodium hydroxide

was added until the paper became pink; .5 N hydrochloric acid was then added drop by drop until the paper took the color of a similar piece in the phthalate buffer. Ten ml. of the buffer were added and the solution diluted to about 40 ml. Five ml. of ferron reagent were next added and the solution made up to 50 ml. Turbid solutions obtained occasionally were filtered through dry crucibles. Transmittance measurements were made 30 minutes later at 620 mμ, and the result of the analysis was read from a graph prepared with standard solutions made in the same manner as the test solutions and containing the same amounts of orthophosphate, added as standard potassium dihydrogen phosphate solution. Readings taken 12 to 24 hours later practically always checked the initial readings closely.

Swank and Mellon (1937) state that the transmittance-concentration curves for this determination do not follow Beer's law, for the conditions used in their studies. Under the conditions outlined above, with solutions 1.308 cm. in thickness, the calibration curves followed Beer's law for concentrations between 0 and .5 milligram of iron per 50 ml., the slope varying somewhat with the orthophosphate concentration.

Mercaptoacetic Acid Method: The test solution was neutralized to Congo red paper with 4 N aqua ammonia. Ten ml. of the reagent were added, then 2 ml. of 4 N aqua ammonia, and the solution was made up to 50 ml. The precipitate which formed was centrifuged down and when necessary filtered off with a dry Pyrex filtering crucible. After 30 minutes the transmittance was measured at 535 mμ, and the result of the analysis read from a graph prepared with standard solutions made in the same manner as the unknowns, without phosphate additions. Beer's law held over the range from 0 to .5 milligram of iron per 50 ml.

Dipyridyl Method: A five-gram sample was mixed in a centrifuge tube with 10 ml. of .6 N trichloroacetic acid, covered, and allowed to stand overnight. In some cases the entire extract was then separated by several centrigual washings, concentrated by boiling, filtered, neutralized, buffered, divided into two portions, and analyzed as described below. A simpler procedure was also found to give the same results. After the overnight digestion, 10 ml. of water were added and the mixture was stirred thoroughly and centrifuged. The largest convenient aliquot of the supernatant liquid was then filtered and neutralized to Congo red paper with aqua ammonia. Ten ml. of acetate buffer were added and the solution was made to 50 ml. and divided into two 25-ml. portions. One hundred milligrams of sodium thiosulfate were added to one portion and after 10 minutes it was made up to 50 ml. with five ml. of .2 per cent dipyridyl reagent and distilled water. The other portion was made up similarly without the dipyridyl, as a reference solution. Failure to cancel out the effect of colored and colloidal materials with such a reference solution was found to introduce very large errors. To avoid heating, which develops the color rapidly but sometimes causes formation of colloidal sulfur, the solutions were allowed to stand overnight before testing; it was later found that readings could be made in 30 minutes by substituting one ml. of one-per cent sulfur dioxide solution for the thiosulfate. The transmittance of the pink solution relative to the reference solution was measured at 510 mμ, and the result of the analysis read from a graph prepared with standard solutions made in the same

manner as the unknowns, using water as a reference liquid. The calibration curves followed Beer's law for concentrations between 0 and .25 milligram of iron per 50 ml.

DISCUSSION OF RESULTS

From the average results (Table 1) it is evident that essentially correct values for total iron are obtained both by the ferron and mercaptoacetic acid methods, when analyses are made by Procedures 3 to 6, inclusive. Slightly low values obtained by Procedures 4 and 6 probably represent loss of iron during ignition. Wet ashing is thus preferable, but organic matter resisting attack by the acid mixture must eventually be destroyed if total iron is to be found.

Dried residues will usually contain pyrophosphate, and in analyses made by the ferron method this must be hydrolyzed to orthophosphate. The serious errors caused by neglect of this precaution are shown in the results

TABLE 1

Summary of Iron Determinations

Diet	Milligrams of iron per 10-gram sample						
	Available iron		Total iron				Faulty
	Method 1[1]	7[2]	3[1]	4[1]	5[3]	6[1]	2[1]
1	0.95	0.94	1.55	1.43	1.53	1.50	0.35
2	0.16	0.14	0.33	0.33	0.34	0.32	0.13
3	0.14	0.13	0.32	0.30	0.31	0.29	0.21

[1] Reagent used was ferron. [2] Reagent was mercaptoacetic acid. [3] Reagent was $\alpha\alpha'$-dipyridyl.

obtained by Method 2. In analyses completed by the mercaptoacetic acid method the results of Swank and Mellon (1938) indicate that the presence of orthophosphate and pyrophosphate may in most cases be disregarded. This is also true for the dipyridyl method. Our results show that one principal disadvantage of the mercaptoacetic acid method may sometimes be avoided; the precipitate which forms when the solution is made ammoniacal may be removed by centrifuging or filtration and a correct result obtained by photometric analysis of the clear solution. In view of the ease with which ferric hydroxide dissolves in the diluted mercaptoacetic acid reagent there is very little danger of losing iron in the precipitate, even in the ammoniacal solution. Preliminary separation of the iron is thus not always necessary, although Jackson (1938) has shown that it is advisable when high precision is desired.

The results obtained by Procedure 1 are of particular interest. They show that even after perchloric acid ashing a considerable part of the iron does not react when the usual colorimetric methods are applied to the untreated acid solution, a fact which might well account, in part, for such discrepancies as those pointed out by McCance (1939) in various measurements of total and available iron in foods. In view of the nature of the residues obtained on evaporation to dryness, there is little doubt that this portion of the iron is held in very firm organic combination. Since different samples of the same diet analyzed by Method 1 always gave practically the same result regardless of variations in digestion time, it seems reasonable

to conclude tentatively that determinations by this method may be significant as a measure of "available" iron. The analyses made by the dipyridyl method—which various investigators claim yields available iron—lead independently to the same conclusion, and strongly suggest the possibility of establishing a convenient method for determining both total and available iron with aliquots from the same perchloric acid digestion.

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MICROSCOPIC STUDIES OF TISSUE OF FROZEN FRUITS AND VEGETABLES

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(Received for publication, May 26, 1941)

It is well known that fruits and vegetables are badly damaged by slow freezing but that they can be preserved in a more desirable state if quickly frozen at a low temperature—Woodroof (1938). The latter fact is made use of by the various companies which market frozen foods, and also by home producers who freeze their own garden truck at the freezer-storage lockers so numerous in some sections of the country.

During this investigation tissues were examined which were frozen at the temperature often used in locker plants and also at a much lower temperature. An attempt was made to determine whether the damage characteristically produced by freezing is due to the formation of ice crystals solely or whether some cell rupture is brought about by a portion of the dissolved gases being forced out of solution upon freezing. The effect of freezing in an atmosphere of nitrogen and of carbon dioxide was also investigated.

EXPERIMENTAL PROCEDURE

Various products were frozen in two ways: (1) at low temperature, approximately —31°C.(—23.8°F.), the temperature used in many locker plants, and (2) on solid CO_2, approximately —76°C.(—104.8°F.).

Sections of both thawed and fresh fruit and vegetable tissues were made and studied under the microscope. In both cases air bubbles could be liberated from the intercellular spaces. The fact that the bubbles were more numerous in the thawed tissue would indicate that some gases had been forced out of the cells during freezing. Pressure on the cover glass released the bubbles into the surrounding water proving that there were gases present and not merely droplets left by melting ice crystals. The bubbles have many times been released from the interior of sections several cells thick; hence they could not possibly have been due to air occluded during preparation of the slides.

Cubes of fresh peach tissue, about three millimeters on each side, were placed in water and held under 25 pounds vacuum for 20 minutes. Sections were cut into the water which had been held under vacuum and then examined under a microscope. No air bubbles were observed. When pressure was exerted on the cover glass to the point where cell rupture occurred, small gas bubbles were seen in the mounting fluid. This showed that gas is contained within the cells themselves and not just in the intercellular spaces.

In view of the above, peaches were frozen under 30 pounds pressure to determine whether the increase in the amount of gas present would bring

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about more breakdown in the tissue. For this study both whole and sliced peaches were frozen in gas-tight containers in the presence of either nitrogen, carbon dioxide, or air. Those frozen in the presence of nitrogen or carbon dioxide were placed in the cans, the air immediately evacuated, and into Series A one atmosphere of the desired gas was introduced. Thirty pounds of gas was introduced into Series B. Two similar series, at these pressures, were also frozen in the presence of air. Both series of fruits were frozen in the two ways mentioned above, viz., at —31 and —76°C. These different gases were used to determine their effect on the oxidative enzymes responsible for discoloration of the peach tissue.

In the course of this study, photomicrographic records have been made of damage sustained by different products owing to freezing. The relation-

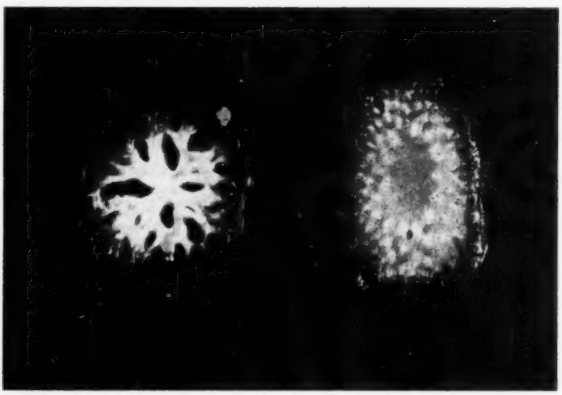

FIG. 1. Mary Washington asparagus. Cross sections of stalks showing effect of freezing at —31°C. (left) and of freezing on solid CO_2 (right) (×2).

ship between tissue injury and freezing temperature has been an interesting aspect of these records.

DISCUSSION

The peaches which were frozen under pressure showed, on the whole, no more cell rupture, and in some cases even less, than those which had not been subjected to this treatment (Plate II, 3-4.)

In the light of this investigation it appears that dissolved gases which are forced out of solution by freezing are in no way responsible for cell rupture, but that this is brought about entirely by ice-crystal formation.

Asparagus frozen at low temperature showed a great amount of cellular breakdown, while that frozen on solid carbon dioxide is a very acceptable product (Fig. 1, Plate I, 1-4). Strawberries frozen whole exhibited cell structure which was much better preserved by freezing on "dry ice" (Plate II, 1-2). Sliced peaches frozen on solid carbon dioxide were superior to those frozen at —31°C. (Plate I, 5-6).

One of the most significant results obtained from the work on pressure and gases was the effect of the gases on color preservation. Heretofore it has been found necessary to freeze peaches and other fruits in a sugar

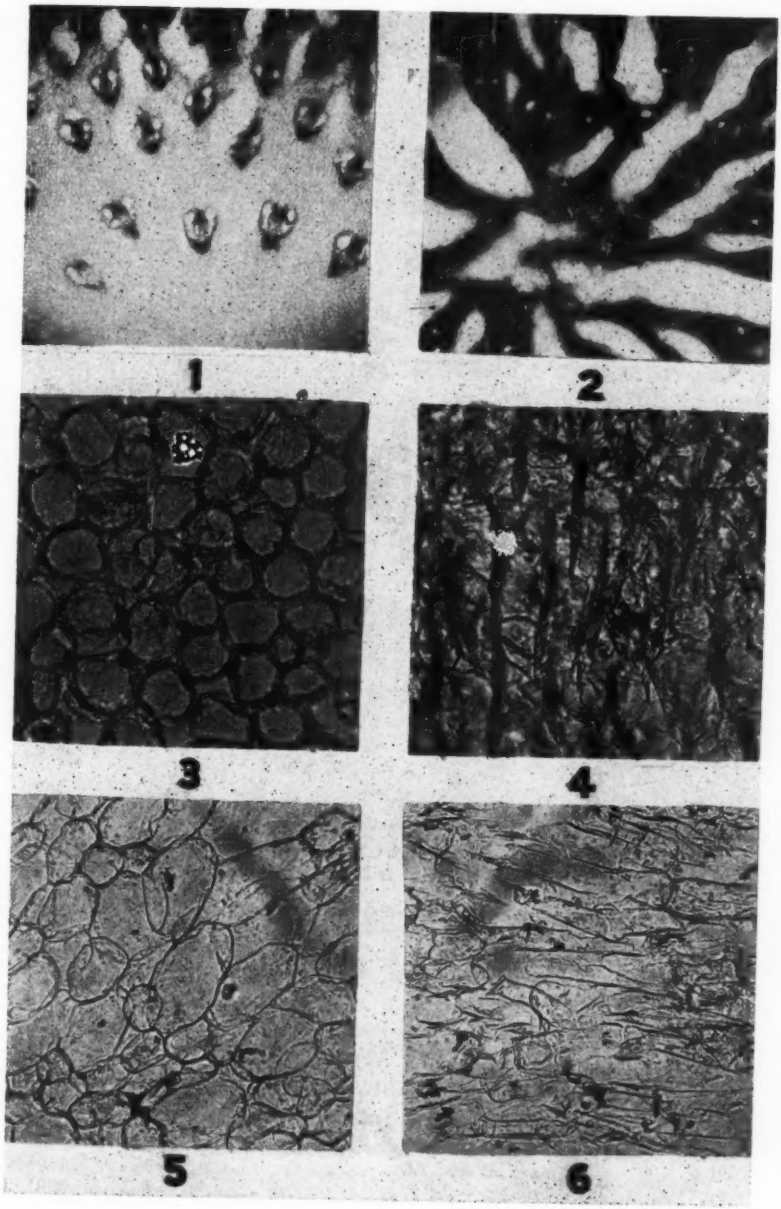

PLATE I

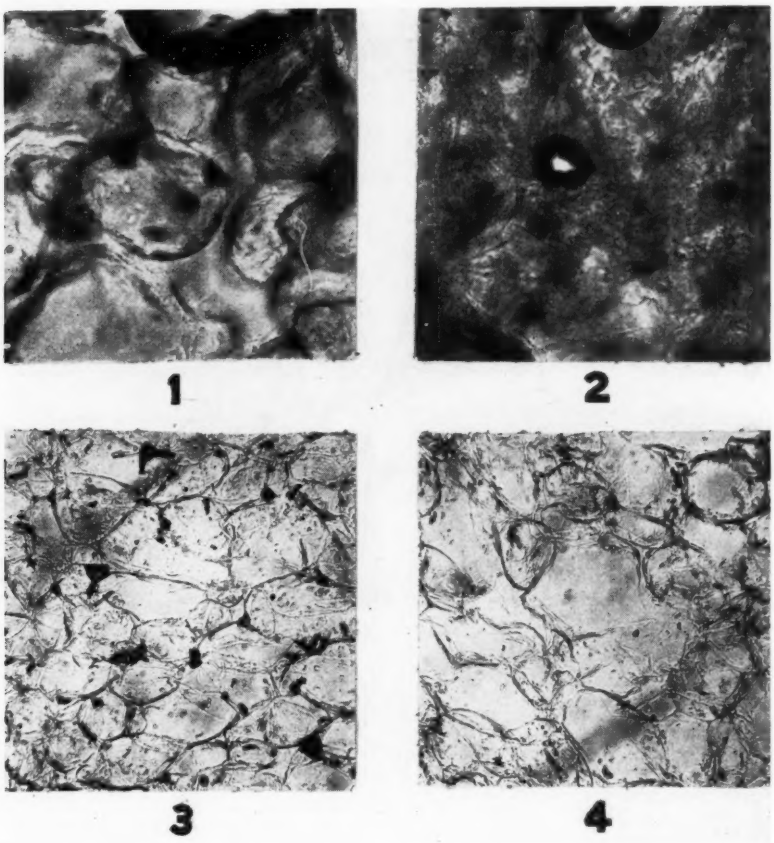

PLATE II
Effect of Freezing at Different Temperatures

PLATE I, 1-4. Cross sections of Mary Washington asparagus, packed in stalks.
1. Frozen on solid CO_2 (× 160)
2. Frozen at —31°C. (× 160)
3. Frozen on solid CO_2 (× 360)
4. Frozen at —31°C. (× 360)

5-6. Cross sections of Elberta peaches.
5. Frozen on solid CO_2 (× 120)
6. Frozen at—31°C. (× 120)

PLATE II, 1-2. Cross sections of strawberries frozen whole in sucrose solution.
1. Frozen on solid CO_2 (× 360)
2. Frozen at —31°C. (× 360)

3-4. Longitudinal sections of Elberta peaches, frozen whole on solid CO_2.
3. Under two atms. of pressure (× 120)
4. Under one atm. of pressure (× 120)

solution in order to prevent oxidation and subsequent loss of color—Pennington (1939) and Tressler and Du Bois (1940). In this experiment when the fruit frozen in the presence of nitrogen was opened after storage for one to five months, it had the color of fresh sliced peaches. The color of this fruit was much better than that of the peaches frozen whole and unpeeled. The cellular breakdown in the sliced fruit was also a great deal less than that in the whole peaches. This is as would be expected, since the more rapid the freezing the better the frozen product—Tressler and Du Bois (1940) and Woodroof (1938). Naturally the slices would freeze quicker than the larger whole fruits.

The fruit frozen in the presence of carbon dioxide gave an acceptable product but was inferior to that which was exposed to nitrogen. That frozen in the presence of air gave a very poor product.

When fruit frozen in one atmosphere of carbon dioxide, nitrogen, or air was removed from the container and allowed to thaw in the presence of air, discoloration was brought about, and the product was also very mushy. If the frozen fruit was allowed to thaw in a thin sugar solution (35 per cent) the fresh fruit color was retained and the texture was firm. Very excellent flavored pies in which the texture and color of the fruit were similar to those of fresh peaches were made from this fruit.

Although the results of the use of increased pressure are inconclusive, the tissues of the fruit held under 30 pounds pressure was, on the whole, better than that stored under only one atmosphere (Plate II, 3-4). In view of this fact further experimentation is planned in which higher pressures will be used.

SUMMARY

The color and texture of peaches can be preserved by quick-freezing the sliced fruit in an atmosphere of nitrogen even in the absence of syrup.

In the cases studied it appears that cellular breakdown is due entirely to ice-crystal formation, and not to the forcing out of solution of gases upon freezing.

As yet there are no conclusive results on the effect of increased pressure on fruits during freezing, but the evidence indicates that increased pressures may be advantageous (Plate II, 3-4). Further studies are to be made in this regard.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. G. F. Smith, Professor of Chemistry, University of Illinois, for his coöperation.

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VITAMIN C, CAROTENE, CALCIUM, AND PHOSPHORUS IN EXPRESSED VEGETABLE JUICE

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This study was undertaken to determine the proportion of vitamin C, carotene, calcium, and phosphorus in the juice expressed from celery, cabbage, carrots, spinach, and green beans with the aid of the Health Mine Juice Extractor, an inexpensive, metal, hand-turned juicer which is being sold to householders. The effect of standing on the two vitamins was also investigated. Quantitative chemical tests for the nutritional elements were run on the juice and the results compared with values obtained from similar determinations on the whole raw vegetable. The data concerning total yields of the juices or nutritive elements are not considered to apply to commercial juicing processes in which extraction is no doubt more uniform and more complete, thus giving better yields of juice, and also more rapid, favoring better immediate yield of ascorbic acid. With the apparatus used in this study the juicing time was as short as any efficient user could make it, being in general three to five minutes for two to four ounces of juice. Possibly motor-driven household juicers would give results comparable to the commercial process.

EXPERIMENTAL PROCEDURE

Portions of the juice were analyzed at once, other portions were allowed to stand for one-half hour, one hour, or overnight. Vitamin C was extracted by the Maclinn and Fellers' (1938) modification of the Bessy and King (1933) method in which three per cent trichloracetic and two per cent metaphosphoric acids were used, and titrated with indophenol standardized by the method of Menaker and Guerrant (1938); carotene was determined by the method of Peterson, Hughes, and Payne (1939) except that the separation of biologically inactive carotenoids from the Skellysolve B phase was made with aqueous diacetone, according to Hegsted, Porter, and Peterson (1939), and the final determination of the purified pigments in Skellysolve was made colorimetrically by comparison with .036 and .1 per cent potassium dichromate solutions; calcium was determined by the Shohl and Pedley (1922) modification of the McCrudden (1911) method; and phosphorus by the Fiske and Subbarow (1925) method.

It was interesting to note that although the water content of all these vegetables is around 90 per cent. the amount of juice expressed with the particular juicer used, varied greatly and was in some cases quite small. The yield from celery was 57 to 71 per cent, from cabbage 50 to 78 per cent, from green beans 40 to 48 per cent, from spinach 35 to 58 per cent, and from carrots 20 to 33 per cent. The smaller samples usually gave the lower percentage yields.

TABLE 1

Reduced Ascorbic Acid and Carotene Content of Whole Vegetables and Expressed Juice[1]

Vegetable	Vegetable[2]		Juice[3]		Per cent of vitamins in juice	
	Reduced ascorbic acid	Carotene	Reduced ascorbic acid	Carotene	Reduced ascorbic acid	Carotene
	mg.	*mg.*	*mg.*	*mg.*	*pct.*	*pct.*
Celery	5.25	.....	0.425	.....	8	....
Cabbage	32.9	.....	22.7	.....	69	....
Carrots	4.24	4.7	1.11	1.05	26	22
Spinach	26.7	5.3	9.71	1.86	30	36
Green beans	.....	0.44	.....	0.104	....	25

[1] Computed on the basis of 100 grams for whole vegetable. [2] All values given are averages of those obtained on three separate samples from one lot of vegetables. [3] Juice expressed directly into mixture of three per cent trichloracetic and two per cent metaphosphoric acids.

TABLE 2

Calcium and Phosphorus Content of Whole Vegetables and Expressed Juice[1]

Vegetable	Vegetable[2]		Juice[3]		Per cent of minerals in juice	
	Calcium	Phosphorus	Calcium	Phosphorus	Calcium	Phosphorus
	mg.	*mg.*	*mg.*	*mg.*	*pct.*	*pct.*
Celery	31.9	29.1	28.7	18.1	90	62
Cabbage	36.8	25.5	10.8	9.30	29	36
Carrots	31.3	20.7	7.8	5.01	25	24
Spinach	142.0	25.6	21.7	6.56	15	26

[1] Computed on the basis of 100 grams for whole vegetable. [2] All values given are averages of those obtained on three separate samples from one lot of vegetables.

TABLE 3

Effect of Standing on Reduced Ascorbic Acid and on Carotene in Juices[1]

Vegetable	Reduced ascorbic acid tested[2]		Carotene tested[2]		
				After standing	
	At once[3]	After standing one hour	At once	30 minutes	Overnight in refrigerator
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Celery	0.425	0	.....	.....	.....
Cabbage	22.7	15.3	.....	.....	.....
Carrots	1.11	0	1.05	1.00	1.07
Spinach	9.71	5.90	1.86	1.95	1.89
Green beans	.....	.....	0.104	0.112	0.115

[1] Computed on the basis of 100 grams for whole vegetable. [2] All values given are averages of those obtained on three separately juiced samples. [3] Juice expressed directly into mixture of three per cent trichloracetic and two per cent metaphosphoric acids.

For the most part the juices were unattractive in color and flavor but it should be stated that the usual recommendations are to mix such vegetable juices with citrus fruit or pineapple juices.

The proportion of nutritional elements expressed in the juice varied widely from vegetable to vegetable (Tables 1 and 2). The vitamin C ranged from eight to 69 per cent, the carotene from 22 to 39 per cent, calcium from 15 to 90 per cent, and phosphorus from 24 to 62 per cent. It is thus evident that if only the juices are used a considerable portion of these elements are lost to the consumer.

The reduced ascorbic acid in celery and carrots had entirely disappeared at the end of one hour, while the amount in cabbage and spinach had decreased appreciably in the same length of time (Table 3). Whether the

TABLE 4

Comparison of Vitamins and Minerals in Equal Weights of Expressed Juices and Whole Vegetables[1]

Vegetable	Vitamin C[2]	Carotene	Calcium	Phosphorus
	mg.	*mg.*	*mg.*	*mg.*
Celery	5.25	.....	31.9	29.1
Vegetable juice	0.701	.....	41.4	26.0
Cabbage	32.9	.....	.....	.....
Vegetable juice	34.3	.....	.....	.....
Carrots	4.24	4.7	31.3	20.7
Vegetable juice	5.28	3.6	24.3	7.73
Spinach	.....	5.3	142.0	25.6
Vegetable juice	.....	3.4	152.0	17.9
Green beans	.....	0.44	.....	.....
Vegetable juice	.....	0.25	.....	.....

[1] Computed on the basis of 100 grams for approximately four ounces. [2] Juice expressed directly into mixture of three per cent trichloracetic and two per cent metaphosphoric acids.

decrease was due to the formation of dehydroascorbic acid alone, or whether further oxidation products had been formed was not determined. Carotene, on the other hand, was not oxidized even on standing overnight.

When the expressed juice and whole vegetables are compared weight for weight as shown (Table 4), it is interesting to note that the two are in some instances of about equal nutritive value. There are, however, several exceptions of which celery juice is the most marked. Its vitamin C content is so low that 730 grams, about seven small glasses would be required to give the vitamin equivalent of 100 grams of the whole vegetable.

SUMMARY

It was found that vegetable juices contain from eight to 69 per cent of the reduced ascorbic acid, from 22 to 36 per cent of the carotene, from 15 to 90 per cent of the calcium, and from 24 to 62 per cent of the phosphorus of the whole vegetable.

In none of the juices studied was there any loss of carotene on standing.

The reduced ascorbic acid content of all of the juices decreased appreciably on standing.

Weight for weight most of the juices are almost as good sources of nutritional elements as are the whole vegetables.

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ASCORBIC ACID CONTENT OF SEVEN VARIETIES OF MUSCADINE GRAPES

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The muscadine grape *(Vitis rotundifolia)* is peculiar to the southeastern coastal plain area of the United States. According to Young (1920) and Dearing (1938), the Scuppernong, Mish, James, Thomas, and Hopkins, five of the seven varieties considered in this study, were discovered in the Carolinas; the Eden was discovered in Georgia and the Labama was introduced in Texas by Dr. T. V. Munson.

The Scuppernong, which probably is the oldest cultivated variety of American grape, was found about 1760 in Tyrrell County, North Carolina, in the vicinity of the Scuppernong River from which the grape derives its name. The Mish, James, Thomas, and Hopkins, which were discovered approximately 100 years later, were named for their discoverers.

Relatively few studies have been published reporting the ascorbic acid content of grapes. More than 20 years ago Chick and Rhodes (1918) showed that the juice of grapes contained about 10 per cent as much ascorbic acid as oranges; Merjanian (1926) reported varying amounts of the vitamin in grapes. Daniel and Munsell (1932a, 1932b) state that 15 grams daily of fresh Sultanina or of Malaga grapes are insufficient to protect guinea pigs from scurvy as determined by the Höjer method, and the Concord showed no evidence of ascorbic acid in the edible portion. Birch, Harris, and Ray (1933) found grapes to contain less than .03 mg. per gram. The grapes assayed by Cartenì (1934) all showed traces of ascorbic acid. According to Morgan, Kimmel, Field, and Nichols (1935) 30 grams daily of Sultanina did not afford full protection to guinea pigs. Levy and Fox (1935) found South African purple grapes to contain $<.01$ mg. of ascorbic acid per gram. Recently, Floyd and Fraps (1939) reported two analyses of muscadine grapes (no variety indicated) as 5.5 and 1.9 and three determinations on Scuppernongs as 7.2 mg. per 100 gm. Kramer and Satterfield (1942) found no ascorbic acid in several samples of muscadine wines.

EXPERIMENTAL PROCEDURE

Through the coöperation of Professors M. E. Gardner and J. G. Weaver of the Department of Horticulture, School of Agriculture, University of North Carolina, seven varieties of muscadine grapes were secured for this study. All varieties were grown on the same type soil (sandy loam with outcrops of clay) and the same fertilizer treatments were given to each. Only firm ripe (or green as desired) fruit of characteristic size for each variety was used for analysis.

The method used for the determination of ascorbic acid was essentially that described by Bessey and King (1933) and modified by Musulin and

King (1936) and Mack and Tressler (1937). From six to eight grapes were cut with a sharp knife so that the pulp and juice could be removed and accurately weighed. The edible portion was ground with iron-free sand and 20 ml. of an acid mixture containing four per cent metaphosphoric acid and eight per cent acetic acid. The extraction was repeated and then the pulp was washed twice with 10-ml. portions of the acid mixture. The extracts and washings were made up to 100 ml. with the acid mixture. A five-ml. aliquot was pipetted into a No. 3, Diamond I capsule vial and titrated with 2,6-dichlorophenolindophenol (21 mg. in 100 ml.), using a modified Neale-Forbes Titration Assembly according to the technique of

TABLE 1

Ascorbic Acid Content[1] *of Seven Varieties of the Muscadine Grape (Edible Portion)*

Variety						
Scuppernong	Labama	Eden	Thomas	James	Mish	Hopkins
mg./100 gm.	*mg./100 gm.*	*mg./100 gm.*	*mg./100 gm.*	*mg./100 gm.*	*mg./100 gm.*	*mg./100 gm.*
8.2	6.3	5.7	5.9	5.5	1.8	<0.2
7.7	6.2	5.6	5.9	5.1	1.7	<0.2
7.6	6.2	5.6	5.8	5.0	1.7	<0.2
7.5	5.8	5.2	5.8	4.9	1.6	<0.2
7.5	5.7	5.2	5.7	4.7	1.5	<0.2
7.4	5.5	5.1	5.2	4.7	1.5	<0.2
7.2	5.5	5.0	5.1	4.6	1.3	<0.2
6.9	5.4	5.0	4.4	4.5	1.2	<0.2
6.7	5.3	4.9	4.3	4.2	1.2	<0.2
6.7	5.3	4.8	4.2	3.8	1.1	.....
6.6	5.3	4.8	4.1	3.8	1.0	.....
6.4	5.2	4.3	3.9	3.7	1.0	.....
6.4	5.2	4.3	3.1	2.6	1.0	.....
4.8	5.0	4.3	3.0	2.5	1.0	.....
4.6	4.8	4.0	2.9	2.0	0.8	.....
Av. 6.8	5.5	4.9	4.6	4.1	1.8	<0.2

[1] By analysis of variance a highly significant difference was shown, F = 79.7 (3.25**). By student's "t" test the least significant difference between varietal means was .86 (omitted Hopkins in the statistical analysis).

Satterfield, Perlzweig, and Dann (1936). Pure ascorbic acid was used to standardize the dye.

The edible portion and the skins (hulls) of both ripe and green Scuppernong grapes were assayed for ascorbic acid as described above.

DISCUSSION

The ascorbic acid content of seven varieties of muscadine grapes grown during the 1937,[1] 1938, and 1939 seasons (Table 1) show definite varietal variations. The Scuppernong shows the highest ascorbic acid content, averaging 6.8 mg. per 100 gm., whereas the Labama, Eden, Thomas, and James average from 5.5 to 4.1 mg. per 100 gm. The Mish and the Hopkins contain negligible quantities of ascorbic acid since they average 1.8 and <.2 mg. per 100 gm., respectively. It should be of interest to note that the Scuppernong

[1] The Scuppernongs were produced in 1937; the Labama and Eden in 1938; the Thomas, James, Mish, and Hopkins in 1939.

is approximately 35 times as rich as the Hopkins and almost four times as potent as the Mish in ascorbic acid.

The age of the vines probably has little or nothing to do with the ascorbic acid content, since the Hopkins and Scuppernong vines are about the same age and show the greatest differences in the vitamin content. It may also be noted that the Scuppernong is the oldest of the varieties and contains the largest amount. Only one of the varieties considered in this study, the Labama, was produced by crossbreeding and it is second to the Scuppernong in ascorbic acid content.

The skins of both green and ripe Scuppernong grapes have about three times the ascorbic acid content of the edible portion. For green grapes the skins averaged 20.2 and the edible portion 6.7 mg. per 100 gm., while for ripe grapes the skins averaged 18.4 and the edible portion 6.8 mg. per 100 gm. Similar results have been reported on other fruit; Levy and Fox (1935) found more ascorbic acid in the skin than in the meat of apples. Likewise, Giroud, Ratsimamanga, Leblond, Chalopin, and Rabinowicz (1936) reported more in the peel than in the pulp of oranges; and Rudra (1936) reported that both pears and plums contain more ascorbic acid in the skin than in the meat. On the other hand, Fujita and Ebihara (1937) showed that figs have more ascorbic acid in the inner meat than in the outer and Clegg and Satterfield (1940) obtained similar results in watermelons. One will observe that the ascorbic acid content of the edible portion of green and ripe Scuppernongs are essentially the same. Carteni (1934) obtained similar results with Italian grapes.

If, as King (1941) suggests, an adult requires 70 to 75 mg. of ascorbic acid daily, two and one-half pounds of Scuppernong grapes would be necessary to meet the need.

SUMMARY

The ascorbic acid content of seven varieties of muscadine grapes is shown.

The average ascorbic acid content of the Scuppernong is 35 times as great as the average for the Hopkins.

The skins of the Scuppernong contain three times as much ascorbic acid as the edible portion.

Green and ripe Scuppernongs show the same quantity of ascorbic acid.

The muscadine grape is not a rich source of ascorbic acid.

ACKNOWLEDGMENT

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PALATABILITY AND HISTOLOGICAL CHANGES OCCURRING IN NEW YORK DRESSED BROILERS HELD AT 1.7°C.(35°F.)[1]

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Since little has been published concerning the optimum time for storage of poultry before consumption, this study was undertaken to determine some of the changes which occur in New York dressed broilers held at 1.7°C.(35°F.). The optimum storage time would be that period which would permit the greatest increase in tenderness without noticeable deterioration in the flavor and aroma of the chickens.

Nickerson and Fitzgerald (1939) made an investigation of the changes taking place in uneviscerated chickens held under refrigeration. They found that gall stains occurred in a small proportion of chickens that were refrigerated and held undrawn for a short time. They concluded that the stains were due to the infiltration of gall, and they were present only when the gall bladder was lying next to the flesh. In most cases the stains were found only in those tissues which were directly against the gall bladder. The portions stained with gall were very bitter, but this was not true for the surrounding tissues.

Fitzgerald and Nickerson (1939) studied the influence of time and temperature of holding undrawn poultry upon its quality. They found that chickens could be held eight days without the development of off-flavors provided they were killed, dressed, and refrigerated properly within 70 minutes after killing. They reported that under these conditions the gizzard may become discolored. At ordinary refrigerator temperatures they found no marked loss of flavor, but some localized off-flavors developed. Maximum tenderness occurred in chickens which were held for at least two or three days at temperatures near freezing.

Sair and Cook (1938) studied the development of visceral taint in dressed poultry. They decided that the development of taint was a question of the time the birds were held above the freezing point. They state that poultry stored at 0°C.(32°F.) for six days, or even longer, is generally considered good in eating quality, but this treatment resulted in the most intense odor recorded.

Very few histological studies have been made on poultry. Koonz and Ramsbottom (1939) have reported a method for studying the histological structure of frozen meat. Pennington, Hepburn, St. John, and Witmer (1917) made one of the early studies of the changes in poultry held at temperatures above freezing. For their three experimental temperatures they

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used room temperature, 23.9°C.(75°F.); refrigerator temperature, 7.2 to 12.8°C.(45 to 55°F.); and chill room temperature, 0°C.(32°F.). They found that at all temperatures of storage the muscle cells ruptured and the contents of the cells exuded. These changes seemed to be affected by both time and temperature of holding.

Some important histological work has been published very recently by Carey (1940a, b) in connection with his studies on striated muscle in frogs. His explanations and photomicrographs of the nodal multiplication of striae in heat rigor of striated muscle are particularly noteworthy. He states that in heat rigor of short duration, nodes and internodes can be seen in the muscles under the microscope. The characteristics of the nodes and internodes are described as follows:

"The pressure nodes of the striated fiber contraction waves have deep differential chemical staining, wide diameter, condensed cytoplasm, and rounded, compressed nuclei, multiplication of fine striae and an increase of anisotropy and mineral ash content. The tension internodes stain relatively less deeply and have narrow diameters, rarefied fibrillated cytoplasm, greatly elongated nuclei, coarse striae and a decrease of anisotropy and mineral ash content."

The origin of the very fine striae from the cleavage of the large striae of the internodes can best be seen when the nodes are obliquely placed or when they extend only part of the distance across the width of the muscle fiber. The author states:

"The cross striae in muscle fibers are multiplied in number by optimum heat. They are subtracted through irregular replacement by granules when the heat is adequately lowered over a specific period. The numbers of light and dark cross striae appear to be related to an optimum range of temperature and rate of chemical reaction."

EXPERIMENTAL PROCEDURE

Storage: A total of 34 Leghorn broilers were killed, and all except the controls were cooled in 0 to 2.2°C.(32 to 36°F.) water spray to temperatures varying from 32 to 36°F. The controls were eviscerated and placed in the oven within six to 40 minutes after killing. The remaining birds were stored from 40 minutes to 118 hours before evisceration and cooking. One New Hampshire fryer was killed and cooled in a refrigerator at a temperature of 3.9°C.(39°F.). This bird was never cooked but was held for 118 hours and used only for histological studies.

Cooking: In preparation for cooking, the birds were weighed, eviscerated, and the backbones were removed. They were washed in a running stream of water, dried lightly on a towel, and reweighed. Observations were recorded concerning the appearance, firmness of flesh, discoloration, and aroma of the uncooked bird. A thermometer, with a short bulb and total length of six inches, was inserted into the center of the right thigh parallel to the breast bone. Each bird was placed on a wire rack about one-half inch above the bottom of a shallow, uncovered pan.

Because it was easier to standardize the procedure and regulate the temperature, the birds were cooked in the oven at 150°C.(302°F.) instead of in the broiler. They were cooked with the skin side up for 30 minutes. They were then removed from the oven, and the skin side was turned down in the pan. The aroma during cooking was recorded at this time. The birds were returned to the oven and cooked until the thermometer in the thigh

registered 85°C.(185°F.). The cooked weight of the bird and the weight of drippings were recorded, and the cooking losses were calculated.

The uncooked liver and gizzard were scored for discoloration and aroma. Any gall bladder stain was removed. The liver was cooked for 25 minutes in 61 grams of water in the top of a double boiler. The gizzard was cooked similarly for 45 minutes in 122 grams of water.

Scoring: Aroma, flavor, tenderness, and juiciness were the factors considered in scoring palatability. Three judges rated the desirability of these four factors. The right thigh and right breast muscles as well as the liver and gizzard were cut into three pieces. Each scorer always received the same portion from each of these pieces.

Histological Method: Microscopic studies were made of the muscle fiber and connective tissue of both cooked and uncooked chickens. Small sections of the muscles were fixed in 10 per cent formalin for 24 hours. The dioxan technique was used for dehydrating and clearing. After infiltration with paraffin, the tissues were imbedded in clean paraffin and cut 10 microns thick with a rotary microtome. Tissues were stained on the slides using Weigert's modified stain, Harris alum haematoxylin, and Van Geissen's mixture of acid fuchsin and picric acid. In the resulting slides the muscle fiber was stained yellow, elastic tissue green or blue green, and collagenous tissue red.

In preparation for the staining of fat, small sections of the muscle were placed in a 10-per cent solution of formalin in physiological salt solution for 24 hours or longer. The tissue was then placed on the freezing plate of the microtome and covered with a few drops of gum arabic solution. After freezing with carbon dioxide under pressure, sections of the tissue were cut 15 microns thick. They were then carried through solutions including scarlet red and alum haematoxylin and mounted in glycerine jelly. After this treatment the muscle fibers were stained blue, the fat red.

DISCUSSION OF RESULTS

Palatability Changes: The New York dressed weights of the leghorn broilers varied from 1,105 to 1,750 grams. The mean weight was 1,516 grams. The New Hampshire fryer weighed 1,465 grams.

There was no noticeable deterioration in the uncooked appearance of any of the birds held approximately 18 hours before eviscerating and cooking. With longer periods of storage the extent of deterioration in appearance varied. It took the form of scald marks, bruises, hemorrhages, and blood clots. After storage periods of 90 hours or longer, the change from the desirable fresh appearance was very noticeable.

It was found that the development and passing of rigor varied widely in individual birds and also in different muscles in the same bird. In one bird rigor had passed from the muscles after one hour and 40 minutes of storage (or had never developed), whereas two birds were still in rigor after nearly 15 hours of storage. In the last two instances rigor had passed during preparation for the oven.

That heat, in general, accelerates the passing of rigor is shown by the data of this study. The birds placed in the oven within six minutes after

killing developed rigor during cooking and were still in rigor after removal from the oven. This was also true for one of the birds held 35 minutes before cooking was started. For the second bird held 35 minutes and for other birds in rigor when placed in the oven, rigor had passed from the muscles by the end of the cooking period.

The odor of the kidney diffused throughout the uncooked bird and was noticeable at the hip joint after approximately 18 hours of storage. It was not noticeable at the wing joints until after 40 hours of storage.

The first indication of gall-bladder discoloration appeared on the liver and right breast muscle after about 17 hours of storage. Bile stains on the gizzard and on the leaf fat were noted after 48 hours, whereas a discolored heart was found after 70 hours of storage. Bile stain increased in extent, intensity, and depth with increased storage times.

The scores of the three judges were averaged to give an indication of the effect of time of storage on the aroma, flavor, tenderness, and juiciness of the breast and thigh muscles. The possible scores ranged from 1 to 10, 10 being the most desirable score.

There was no marked change in flavor and aroma scores until after 18 hours of storage (Fig. 1, A and B). Deterioration in flavor occurred more rapidly in the thigh than in the breast muscles although there was variation in the individual birds. Eight of the nine birds stored 41 to 43 hours prior to cooking scored 8 or above for flavor of the breast muscle, whereas only one bird of this group had thigh muscles scoring 8 or higher.

Both breast and thigh muscles were rubbery, tough, and very difficult to chew if in rigor when taken from the oven. Tenderness of the breast muscles increased rapidly until approximately three hours' storage prior to cooking (Fig. 1, C). With longer storage there was a continued gradual increase in tenderness, but the change was relatively slight. In general, thigh muscles were less tender than those from the breast. In addition tenderness of the thigh muscles increased more gradually than that of the breast muscles. A score comparable to the three-hour figure for the breast was not reached in the thigh until after 42 hours of storage.

The thigh was consistently judged to be more juicy than the breast. Juiciness was unaffected by time of storage (Fig. 1, D).

According to the scores of the judges, the tenderness and juiciness of the liver were unaffected by the length of the storage time. The liver was consistently judged very tender, with scores varying from 7.7 to 10. The greatest drop in aroma and flavor scores of the liver occurred after 20 hours of storage. Tenderness and juiciness of the gizzard were also unaffected by the length of storage time. There was a definite decrease in the desirability of the aroma of the gizzard after about 20 hours of storage and a similar drop in the flavor after only three hours of storage.

The total cooking loss averaged 18.4 per cent. The cooking time averaged one hour and 21 minutes. Longer cooking times were accompanied by greater cooking losses. Cooking loss and cooking time both decreased significantly as the time between killing and cooking increased. These results agree with those of Alexander and Clark (1934) who studied the influence of ripening of lamb on the cooking loss and the cooking time. They found that longer ripening gave decreased cooking shrinkage and shorter cooking

time. As they increased the ripening period beyond two days, they found smaller cooking shrinkages and a more rapid rate of heat penetration.

Histological Changes: 1. Muscle fiber and connective tissue of cooked chicken. The microscopic studies made on the cooked chicken muscles after varying the length of the storage period showed some interesting results.

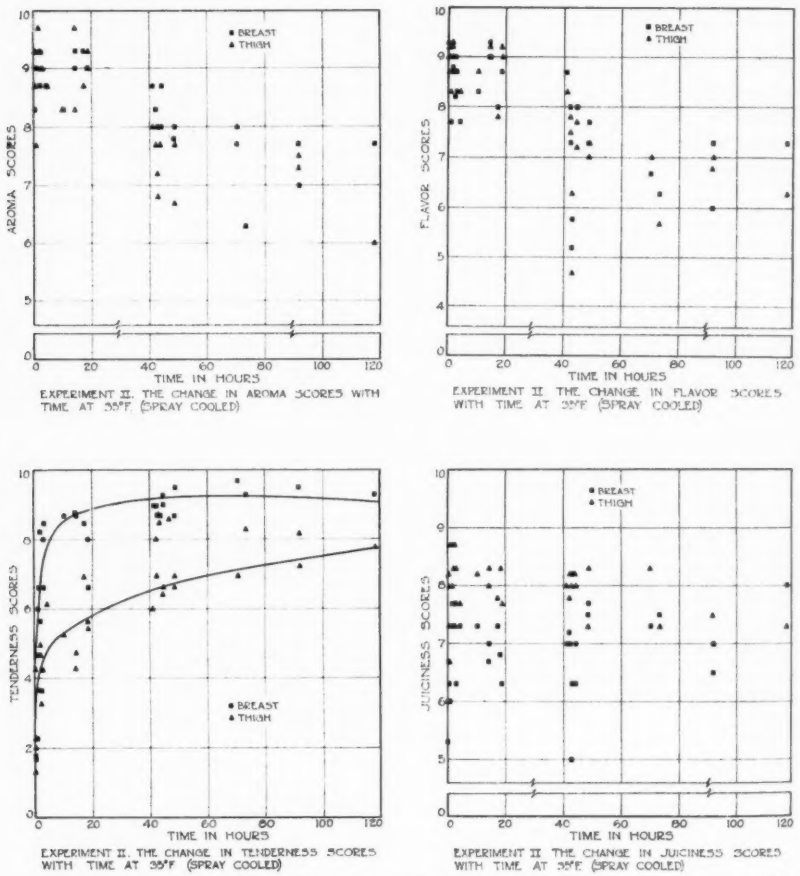
AROMA SCORES
FLAVOR SCORES
TENDERNESS SCORES
JUICINESS SCORES
TIME IN HOURS
BREAST
THIGH
EXPERIMENT II. THE CHANGE IN AROMA SCORES WITH TIME AT 35°F. (SPRAY COOLED)
EXPERIMENT II. THE CHANGE IN FLAVOR SCORES WITH TIME AT 35°F. (SPRAY COOLED)
EXPERIMENT II. THE CHANGE IN TENDERNESS SCORES WITH TIME AT 35°F. (SPRAY COOLED)
EXPERIMENT II. THE CHANGE IN JUICINESS SCORES WITH TIME AT 35°F. (SPRAY COOLED)

FIG. 1. Changes in palatability scores of broilers held for varying periods at 1.7°C. (35°F.) before evisceration and cooking. (Highest possible score, 10.)

Each of the five muscles studied seems to go through similar post-mortem changes, but the changes reach their peak at different times in each muscle. The first change was the appearance of the nodes and internodes characteristic of rigor mortis. Following the passing of rigor, there was a thinning of the cell content at random points in scattered muscle fibers. Then definite breaks in the muscle fiber appeared. The sarcolemma may or may not be broken, but there is a space in the muscle fiber from which the cell contents

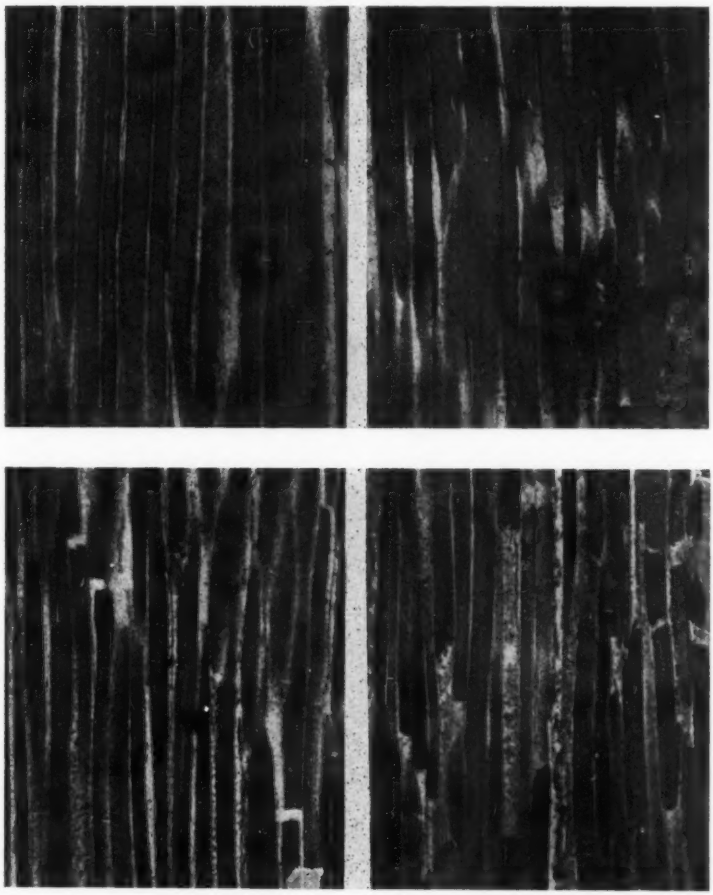

FIG. 2. Photomicrographs showing the effect of storage prior to cooking upon fibers of breast muscle (pectoralis secundus). First thin places appear in the fibers, next definite breaks, and lastly the change from striated to granular-like structure.

A. In oven 6 minutes after killing
B. Storage time, 40 minutes
C. Storage, 2 hours and 40 minutes
D. Storage, 118 hours

Cooked muscle

Magnification approximately × 80

appear to have exuded. The breaks did not seem to occur along any crosswise line of the muscle fiber, since the edges of the breaks were jagged and often occurred obliquely to the lengthwise axis of the fiber. A further disintegration of the muscle fibers, giving the impression of autolysis action, was the change of the muscle contents to a granular type of structure over a relatively long area of the fiber. These changes appeared to be correlated

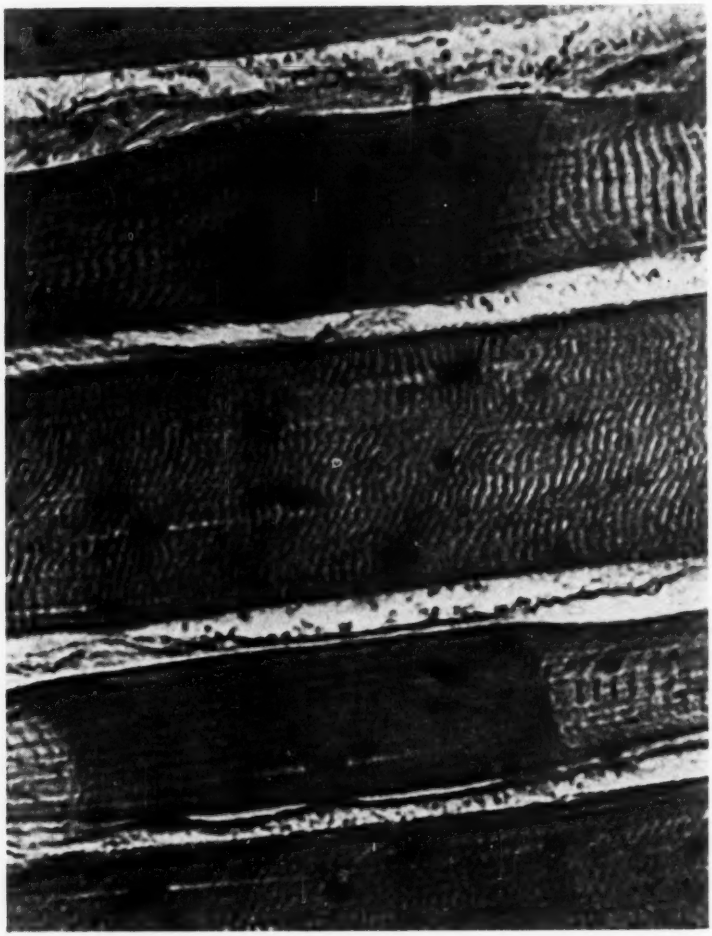

FIG. 3. Photomicrograph showing the nodes and internodes of heat rigor in breast muscle (pectoralis secundus). Photomicrograph of a small section of A, Fig. 2. In oven six minutes after killing.

Cooked muscle Magnification approximately × 825

with changes in tenderness of the muscles. The breaks appeared earlier and were more numerous in the breast muscles than in the thigh muscles. Palatability scores of the judges indicated that the breast muscles became tender sooner than did those of the thigh. Although there were individual differences in the chickens, it is true that in general the birds stored the longest periods showed more disintegration in the muscle fibers.

Two breast muscles (pectoralis major and pectoralis secundus) and three thigh muscles (gluteus primus, semitendinosus, and adductor longus) were

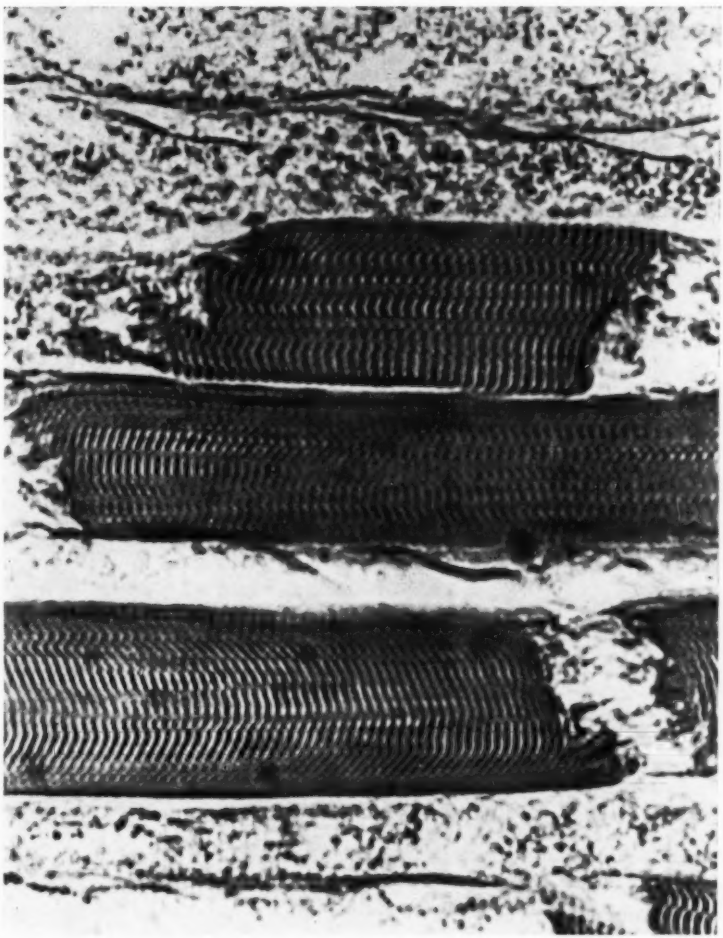

FIG. 4. Photomicrograph showing the change from striated to granular-like structure in breast muscle (pectoralis secundus). Photomicrograph of a portion of D, Fig. 2. Storage time, 118 hours.

Cooked muscle Magnification approximately × 825

studied microscopically. Of these the pectoralis secundus was, according to the judges, the most tender. Microscopic study of its fibers indicated that the post-mortem changes occurred earlier in this muscle than in the others. The chickens which were placed in the oven within six minutes after killing were still in rigor after cooking. A section of the pectoralis secundus from one of these birds showed the nodes and internodes characteristic of rigor (Fig. 2, A). Close examination of the photomicrograph will reveal 10 or more nodes. A greater magnification of some of these nodes shows the rela-

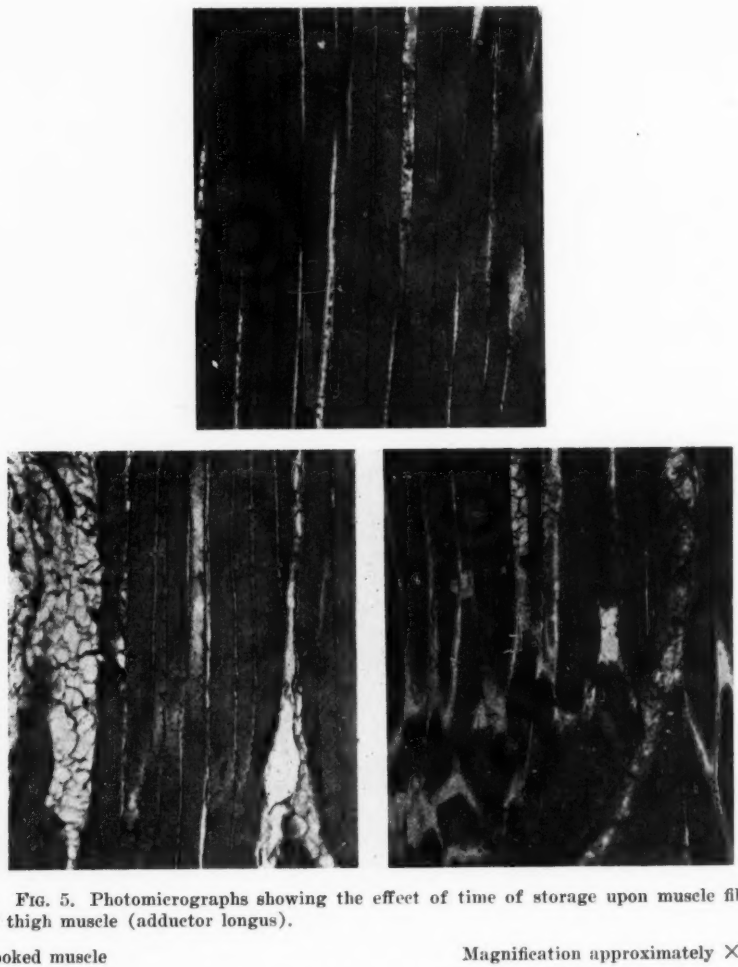

FIG. 5. Photomicrographs showing the effect of time of storage upon muscle fibers of thigh muscle (adductor longus).

Cooked muscle Magnification approximately × 80

A. In oven 6 minutes after killing
C. Storage, 118 hours B. Storage, 49 hours

tively narrow internodes with coarse striations and the swollen nodes with very fine striations (Fig. 3).

After a storage of 40 minutes prior to cooking, the pectoralis secundus showed thin places at irregular intervals throughout the muscle fibers (Fig. 2, B). Following approximately two hours and 40 minutes the typical breaks occurred in the fibers (Fig. 2, C), whereas they became very numerous with 18 hours of storage. After storage for 118 hours, the disintegration or change to the granular-like structure of the fibers was very noticeable (Fig. 2, D). The granular-like structure occurred for long distances in

some fibers, and there were partial or complete breaks in other fibers. A greater magnification shows the granular effect more clearly (Fig. 4).

The changes in the pectoralis major were very similar to those in the pectoralis secundus.

The adductor longus is one of the less tender, inner thigh muscles which contains a large amount of fat. Normal muscle fibers were found in the section of this muscle from the chicken which was in the oven within six minutes after killing (Fig. 5, A). A few thin places in the fibers may be noted in the section taken from the chicken stored about 49 hours, and a few breaks in the fibers were found after storage for 118 hours (Fig. 5, B and C).

The gluteus primus and the semitendinosus follow similar patterns, except that the changes in these muscles occur earlier than is the case with the adductor longus. However, the appearance of the thin places and breaks in the fibers occurred later in the thigh than in the breast. Thin places in the fibers of the gluteus primus were first noted after 10 hours and 30 minutes of storage. The first actual breaks in the fibers were not seen until the chicken had been stored almost 19 hours. The semitendinosus muscle seems to be the most variable of the five studied. There were definite breaks in the fibers of this muscle from a bird stored two hours; however, the section from another bird stored three hours showed only the unchanged muscle fibers. After almost 19 hours of storage there were definite breaks as well as thin places in the fibers. If the thigh muscle fibers developed the granular-like structure characteristic of the breast muscles after 118 hours of storage, it was not observed. Instead of the granular appearance the microscopic study showed nothing in the broken areas except in some instances leucocytes or erthrocytes had fallen into them.

2. Muscle fiber and connective tissue of uncooked chicken. Microscopic studies were made of sections of the uncooked muscles of the fryer after it had been stored for varying lengths of time. From this one study it appears that the toughest muscles went into rigor first, the most tender ones last. One section which appeared to be in rigor was obtained from the pectoralis major after storage for one hour and 30 minutes (Fig. 6, A). An enlarged section is shown in Fig. 7. The disintegration, which was so typical of the cooked muscle, was also noted in the uncooked muscle. With studies made on one chicken, it is not possible to say definitely after what periods of storage the successive changes occur in the five muscles. The evidence does indicate that the changes are not apparent after such short periods of storage as was the case with the cooked muscles. The typical breaks were observed in the fibers of all five muscles after storage for approximately 73 hours, although in some muscles they were noted earlier (Fig. 6, B, C, and D). Since the breaks in the fibers do occur eventually in the uncooked muscles, the influence of heat appears to be a possible explanation for the earlier appearance of the breaks in the fibers of the cooked muscles. The longitudinal and cross striations, which are both typical of fresh tissue, may be noted in the section photographed at high magnification (Fig. 7).

3. Fat distribution. Histological studies showed that there is considerable variation in the amount of fat deposited in the different muscles of

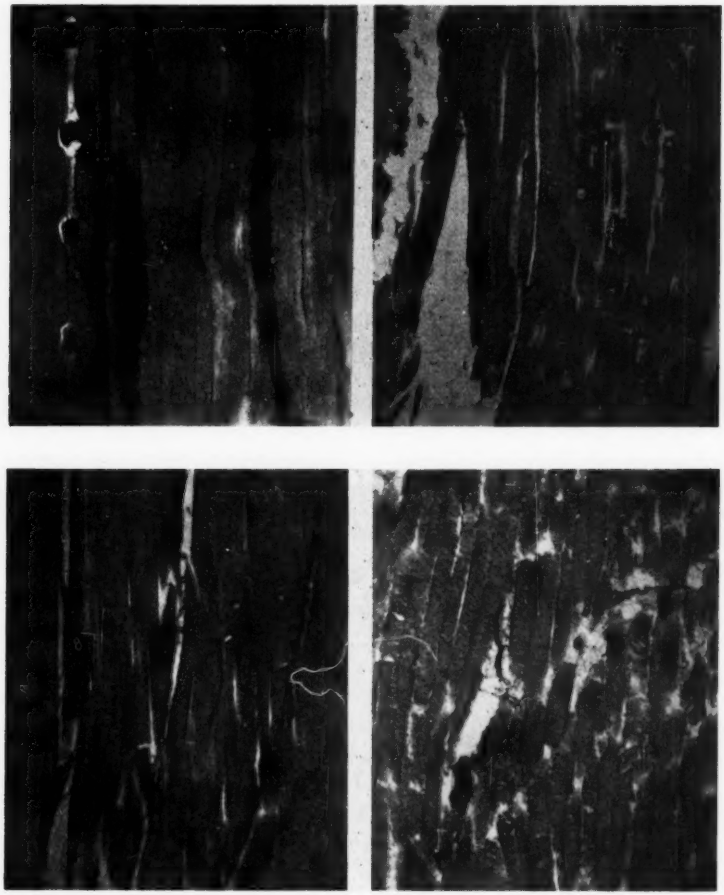

FIG. 6. Photomicrographs showing the effect of storage at 1.7°C. (35°F.) upon muscle fibers. The changes appear more slowly than in the cooked muscle.

A. Storage, 1 hour and 30 minutes
B. Storage, 94 hours
C. Storage, 94 hours
D. Storage, 94 hours

A. Pectoralis major
B. Pectoralis major
C. Gluteus primus
D. Adductor longus

Uncooked muscle

Magnification approximately × 80

the chicken. Thigh muscles, in general, contain more fat than do breast muscles. This probably partially accounts for the greater juiciness of the thigh. Both the pectoralis major and the pectoralis secundus had very little fat. The gluteus primus, the thigh muscle which most closely resembles a breast muscle in color and tenderness, showed somewhat more than the

FIG. 7. Photomicrograph showing the nodes and internodes of rigor in breast muscle (pectoralis major). Both the longitudinal and cross striations of fresh muscle are shown. Enlargement of a portion of A, Fig. 6. Storage time, one hour and 30 minutes.

Uncooked muscle Magnification approximately × 650

breast muscles. The semitendinosus contained more fat than the gluteus primus. The inner thigh muscle, the adductor longus, contained the most fat of any of the muscles studied.

SUMMARY

When New York dressed broilers were spray-cooled and stored at 1.7°C.(35°F.), the first decrease in aroma and flavor scores occurred after 40 hours. In general, this decrease continued thereafter with increase of

storage time before cooking. Tenderness of the breast increased rapidly with storage until approximately three hours. Tenderness of the thigh increased more slowly than for the breast, a score comparable to the three-hour figure for the breast not being reached until after 42 hours of storage. The thigh was consistently judged to be more juicy and less tender than the breast. Juiciness was unaffected by time of storage.

The greatest decrease in the desirability of the aroma and flavor of the liver occurred after 20 hours' storage. The aroma scores of the gizzard dropped after the same period of time, but the decrease in flavor of the gizzard occurred after only three hours' storage. Tenderness and juiciness of the liver and gizzard were unaffected by the length of the storage time.

Microscopic studies of two breast muscles and three thigh muscles showed similar post-mortem changes occurring in each muscle, but the time required for the same degree of change varied considerably for each muscle. The first change was the appearance of the nodes and internodes characteristic of rigor mortis. Following the passing of rigor, there was a thinning of the cell contents at random points in scattered muscle fibers. Then definite breaks appeared in the fibers. Finally there was a change of the striated to a granular type of structure over a relatively long area of the cooked fiber. These changes appear to be correlated with changes in tenderness of the muscles. The post-mortem changes occurred more slowly in the uncooked than in the cooked fibers.

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CHRONIC TOXICITY OF DIPHENYLENE SULFIDE TO THE ALBINO RAT[1]

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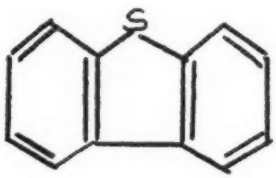
The search, in recent years, for new insecticides has called attention to many aromatic compounds—Fink and Smith (1936), Fink and Vivian (1936), Campbell, Sullivan, Smith, and Haller (1934), and Smith, Siegler, and Munger (1938). Fink and Smith (1936) demonstrated that diphenylene sulfide (dibenzothiophene, biphenylene sulfide, $C_{12}H_8S$), in a concentration of 3.3 parts per million, killed 50 per cent or more of a preparation of Culicine mosquito larvae in 16 hours at 26°C. (78.8°F.). Smith, Siegler, and Munger (1938) noted a similar lethal efficiency of the compound against codling moth larvae. Its larvicidal action was one of the best of over 400 compounds tested by the Bureau of Entomology and Plant Quarantine of the U. S. Department of Agriculture.

Diphenylene sulfide crystals are yellowish white in color and melt at 97°C. (206.6°F.). The compound has the structural formula:



It is insoluble in water, slightly soluble in alcohol, and very soluble in ether and benzene. The compound has a moderately bitter taste and a rather unpleasant odor.

Investigation of the possibility of public-health hazards from potential spray residues is an important part of any program designed to evolve effective substitutes for sprays containing lead, arsenic, and fluorine. Investigation of the possibility of a chronic toxicosis traceable to diphenylene sulfide was, therefore, undertaken at the request of the Insecticide Division of the Bureau of Entomology and Plant Quarantine which supplied the pure compound.

EXPERIMENTAL PROCEDURE

Since a spray residue containing diphenylene sulfide would necessarily enter the body by way of the gastrointestinal tract, small amounts of the compound were mixed with a basic rat diet, the composition of which has been previously reported by Thomas, Wilson, and Eddy (1940). A me-

[1] Agricultural Chemical Research Division Contribution No. 28.

chanical mixer was used for this purpose. Male albino rats, 25 to 28 days of age and averaging 48 grams in weight, were selected and placed in cages in groups of five. Free access to food and water was possible at all times, the food cups being designed to keep food losses negligible. The individual rats and the food cups were weighed twice a week for 165 days, and the average weights of the animals in each group were plotted as shown (Fig. 1).

The concentrations of diphenylene sulfide in the diets fed were .25, .50, and 1.00 per cent during the first four days of the experiment at the end

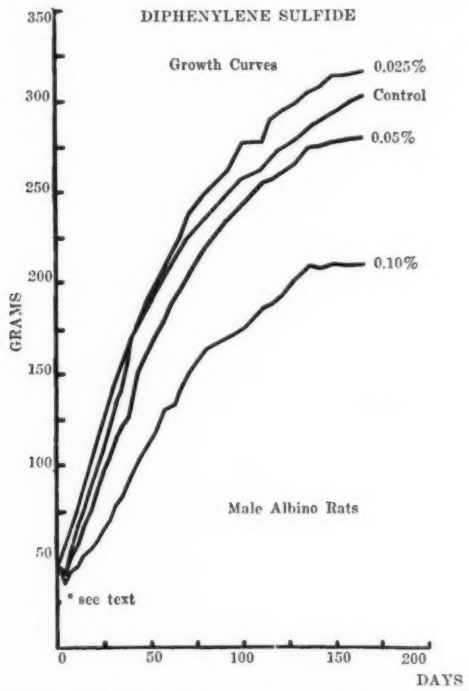


FIG. 1. Effect of diphenylene sulfide on growth rate of albino rats.

of which time they were reduced to one-tenth of these values. The losses in weight of all rats on the experimental diets during these four days are believed to be due to the low food intakes—less than one gram per rat per day in each group. On the highest dosage employed the total consumption of diphenylene sulfide per rat for this four-day period was less than 40 milligrams. Since, as will be shown later, the decreased growth rates are to be attributed largely if not entirely to diminished food intakes, it is believed that the losses in body weight during the first four-day period and the small amounts of diphenylene sulfide ingested had little or no effect on the subsequent course of the growth curves. In harmony with this view is the fact that rapid growth was resumed when the diets containing lower concentrations of the compound were fed. No grossly abnormal appearance

or behavior was noted among the experimental animals throughout the duration of the experiment. The control curve (Fig. 1) is a composite one derived from data accumulated here over a period of several years. The use of such a control curve is justified by the fact that the control average weight-growth curves of the strain of albino rats used in this laboratory have been found to be quite reproducible—Wilson, McNaught, and DeEds (1938).

It will be seen (Fig. 1) that while there was a certain amount of variation in the average weights of the control animals and those receiving the lower concentrations of diphenylene sulfide, no significant effect was obtained until the diphenylene sulfide accounted for .10 per cent of the diet. The fact that the growth curve for rats on a diet containing .05 per cent diphenylene sulfide remains below the control curve, whereas the growth curve for rats on one-half this dosage crosses the control curve before the 50th day and remains above, might suggest a toxic effect on the higher dosage. However, as will be discussed below, this slight effect on growth can be entirely accounted for by the diminution of food consumption.

In studying the chronic toxicity of a given substance ingested daily with a ration known to be adequate for normal growth the determination of growth rates at various dosages of the substance represents the first step in obtaining gross evidence of a deleterious effect. If retarded growth rates are found and the food intakes remain normal, it is evident that retarded growth is due to some specific action of the toxic agent. But if the retarded growth rates are accompanied by decreases in food consumption it then becomes necessary to determine the extent to which diminution of food intake is responsible for retarded growth. The technique of paired feeding experiments serves this purpose and permits a conclusion as to the existence of a specific toxic action apart from inanition.

The essence of the paired feeding technique is the comparison of the growth rate of an animal on an experimental diet with that of an animal receiving an amount of control diet representing a degree of inanition determined by the daily food intake of the animal on the experimental diet.

In the present work paired feeding experiments were not undertaken to determine to what extent growth inhibition should be attributed to lessened food consumption. We may, however, compare the growth rates and food consumptions of the animals receiving diphenylene sulfide with the previously reported growth rates and food consumed by albino rats in the studies on inanition mentioned by Wilson, *et al.* (1938). Such a comparison clearly leads to the conclusion that the lack of growth on diets containing diphenylene sulfide was largely accounted for by the reduction in food intake, and that even on a diet containing .10 per cent diphenylene sulfide the effect attributable to a specific action of the compound was small.

The presentation of a rigorous proof of the correctness of these conclusions would require the reproduction of the growth curves of the present investigation on the same scale and in conjunction with those previously reported on inanition studies, Wilson, *et al.* (1938). This is not justified since the net result of such proof would be that the growth rates and food consumption reveal little of significance regarding the true toxic properties

of diphenylene sulfide as indicated by evidence obtained from histological examination of the tissues of the experimental rats, even those of animals on the lowest dosage level.

AUTOPSY FINDINGS

At the termination of the feeding experiments the animals were sacrificed, the technique of autopsy being that described by Wilson, *et al.* (1938). The inanition studies mentioned above demonstrated that more significance can be given to the figures for the body lengths and organ weights of the albino rat stock in this laboratory if they are compared with those of normal animals of the same weight rather than of the same age. The data for the experimental animals were thus compared with those of control animals of the same weights at autopsy and treated statistically. Since such treatment

TABLE 1

Statistical Treatment of Weights of Livers and Spleens of Control and Diphenylene Sulfide-Fed Rats

Diphenylene sulfide in diet	Mean body weight	Weights of organs with probable errors		Difference A-B	Difference
		A Control rats	B Diphenylene sulfide-fed rats		Probable errors of difference
pct.	*gm.*	*gm.*	*gm.*	*gm.*	
Livers					
.025	310	10.00 ± .11	10.70 ± .29	−0.70 ± .31	2.26
.050	273	9.50 ± .27	12.80 ± .48	−3.30 ± .55	6.00
.100	212	8.40 ± .22	18.10 ± .74	−9.70 ± .77	12.60
Spleens					
.025	310	.97 ± .062	.69 ± .015	.28 ± .063	4.45
.050	273	.92 ± .072	.64 ± .067	.28 ± .098	2.86
.100	212	.83 ± .041	.36 ± .010	.47 ± .042	11.20

of the data revealed no significant changes in body lengths nor in organ weights except in the cases of the livers and spleens, the data (Table 1) are limited to these two organs. The table shows that there was a significant increase in the weights of the livers and a decrease in the weights of the spleens of the rats receiving diphenylene sulfide, these effects being more pronounced as the dosage increased.

The livers of the rats receiving .05 per cent diphenylene sulfide and especially those receiving .10 per cent were found to be unduly large and presented a yellowish, fatty appearance. Except for size the spleens of all animals appeared normal. The gross appearance of all other organs preserved at time of autopsy was normal.

HISTOLOGICAL FINDINGS

Paraffin sections of liver, spleen, adrenal, heart, bladder, intestine, lung, testis, and stomach from five animals on each dosage level of diphenylene sulfide were prepared and stained with hematoxylineosin. Frozen sections of the livers of three of the rats receiving .10 per cent and the livers of all the rats receiving .025 per cent diphenylene sulfide in the diet were made and stained with Sudan IV.

Histological examinations of the livers of the animals which received the .10 per cent dosage revealed extensive fatty metamorphosis of the hepatic cells. In the preparations stained with hematoxylin and eosin a marked swelling and a fine, irregular vacuolation of the parenchymal cells were observed, extending throughout the lobules. In places, indistinct cell borders were encountered so that some adjacent cells appeared fused. In some cells a rim of homogeneous, deeply stained cytoplasm, surrounding groups of vacuoles, was noticed. The cell nuclei were fairly well stained though some were very pale. No definitely necrotic cells were seen. The Kupffer cells had not been altered and there was no evidence of fibrosis. Similar changes, distinctly less marked, but still easily demonstrable were observed in the livers of the animals which had received a diet containing .05 per cent diphenylene sulfide. Still less severe were the structural alterations detected in the livers of the .025-per cent group.

The liver sections stained with Sudan IV showed abnormal fat accumulation. In the liver sections from the rats on .10 per cent diphenylene sulfide diet these fat accumulations, in the form of coarse droplets, were observed in almost every cell. These fat droplets were frequently seen grouped together in one part of the cytoplasm, surrounded by a homogeneous, nonfatty membrane about three micra thick, occasionally resulting in the displacement of the cell nucleus to one side. Two such structures were observed in some cells. Much fat, deposited as above described, was seen in the livers from the rats receiving .025 per cent diphenylene sulfide in the diet, though its distribution was irregular and the total quantity was smaller than in the livers of the animals receiving .10 per cent diphenylene sulfide. Some fairly large groups of cells free from fat droplets were seen in the liver sections of the rats on this lower dosage. There was no demonstrable relationship of the fatty change to the lobule centers.

The kidney sections of animals on all dosage levels of diphenylene sulfide showed a slight to moderate, light brown, granular pigmentation of the epithelial cells of the proximal convoluted tubules, but there was no evidence of cell destruction.

The histology of all the other tissue sections was within normal limits.

BLOOD PICTURE

The blood of five rats receiving .10 per cent diphenylene sulfide in the diet was examined on the 107th and 157th days of the experiment for possible hematoxic effects. Blood samples were obtained from the animals' tails. Hemoglobin was determined in grams per cent, and counts were made of the erythrocytes, reticulocytes, and white cells. Differential counts of the white cells were also made. The values for the five animals agreed well among themselves, the averages being as follows on the 107th day: 16.4 grams per cent hemoglobin, 8,980,000 erythrocytes, 2.42 per cent reticulocytes, and 15,200 white blood cells. On the 157th day of the experiment the values were 17.6, 9,890,000, 2.04, and 14,680, respectively. On the 107th day the differential white cell count was lymphocytes 72.4, polymorphonuclear neutrophils 24.93, monocytes 1.68, basophils .29, and eosinophils .71 per cent. On the 157th day the figures were 63.02, 34.36, 1.37, .56, and .65 per cent, respectively. These figures agree well with those obtained from

numerous counts made in this laboratory on our control animals of similar ages as well as with data quoted by Scarborough (1930-31) and Klieneberger (1927). The counts are also in good agreement with the results previously reported by Thomas, Wilson, and Eddy (1940) on rats receiving diphenylene oxide. As in the case of diphenylene oxide it is concluded that the continued feeding of diphenylene sulfide produced no change in the blood picture of the albino rat.

CHEMICAL EXAMINATION OF THE URINE

The passage of benzene-ring compounds through the animal body frequently results in the introduction of one or more hydroxyl groups, these oxidation products being excreted in the urine as such or in some conjugated form. The conversion of anilin to paraminophenol, Fränkel (1927), and of phenothiazine to hydroxy phenothiazone, DeEds, Eddy, and Thomas (1938), illustrates the process. Accordingly, an attempt was made to isolate an oxidation product of diphenylene sulfide from the urine of rabbits. Rabbits were selected for this purpose because of the necessity for a larger quantity of urine than could be conveniently obtained from rats. The results of a typical experiment were as follows:

An emulsion of two grams of diphenylene sulfide in water was prepared with the aid of powdered gum acacia and administered to a rabbit by means of a stomach tube. The combined urine collections were filtered, and the filtrate extracted with ether. After evaporation of the ether the residue was dissolved in hot water, filtered while hot, and allowed to cool. Upon cooling, long hair-like crystals formed, and upon standing overnight, these crystals changed to shorter needle forms. After recrystallizing three times from water the product was finally recrystallized from 95-per cent alcohol, and had a melting point of 247 to 248°C.(476.6 to 478.4°F.) (uncorrected).

As would be expected if one or more hydroxyl groups had been introduced into one or both of the benzene rings, the product isolated from urine acetylated readily. Acetylation was easily accomplished by heating with acetic anhydride under a reflux condenser for 30 minutes. The acetylated product had a melting point of 208°C.(406.4°F.) (uncorrected). Although the melting points reported herein are uncorrected, they served to differentiate the urine product from its acetylated derivative and as criteria of purity for the two products. For reasons to be given below we believe the product isolated from urine to be a mono-hydroxy-diphenylene sulfone. The only similar compound we have been able to find described in the literature is 3-hydroxy-biphenylene sulfone with a melting point of 264°C.(507.2°F.) —Farbendindustrie (1934).

A sample of the urine product was put through the process of acetylation and subsequent hydrolysis four times to insure purity. The melting point of the unacetylated product remained 247 to 248°C. (uncorrected). Starting with 247 milligrams of material, acetylation and recovery of the derivative were carried out, care being taken to keep the procedure quantitative. The yield of acetylated derivative was 288 milligrams. The theoretical weights of acetylated derivatives, assuming the urine product to be mono-hydroxy-diphenylene sulfide, mono-hydroxy-diphenylene sulfoxide, or mono-

hydroxy-diphenylene sulphone would be 298.9, 295, and 291.7 milligrams, respectively. The observed yield of 288 milligrams is in substantial agreement with the theoretical weight of 291.7 milligrams for the weight of acetylated mono-hydroxy-diphenylene sulfone.

The above conclusion was supported by determination of the amount of acetic acid liberated upon hydrolysis of the acetylated product having a melting point of 208°C. The 288 milligrams of acetylated product were hydrolyzed in half-normal sulfuric acid, and the acetic acid recovered by steam distillation. With phenolphthalein as an indicator the distillate was found to require 10.40 c.c. of N/10 sodium hydroxide for titration. The theoretical amounts of N/10 sodium hydroxide required to titrate the acetic acid liberated by hydrolysis of 288 milligrams of acetylated mono-hydroxy-diphenylene sulfide, mono-hydroxy-diphenylene sulfoxide, or mono-hydroxy-diphenylene sulfone would be 11.95, 11.15, and 10.48 c.c., respectively.

TABLE 2

Analyses of Diphenylene Sulfide Oxidation Product and Its Acetyl Derivative

	Hydrogen	Carbon
	pct.	*pct.*
Oxidation product:		
Calculated for $C_{12}H_8O_3S$	3.48	62.11
Found	3.61	62.30
Found	3.50	61.87
Acetylated oxidation product:		
Calculated for $C_{14}H_{10}O_4S$	3.66	61.05
Found	3.87	61.03
Found	3.93	61.05

Again the last value is in substantial agreement with the observed value of 10.40 c.c. of N/10 sodium hydroxide.

Samples of the pure product isolated from urine and of its acetyl derivative were analyzed for total carbon and hydrogen by Mr. T. H. Harris of the Enzyme Laboratory, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture.

The results (Table 2) show that the analyses check very closely with the predicted formulas and, together with the evidence submitted above, point strongly to the conclusion that passage of diphenylene sulfide through the rabbit results in an oxidation to mono-hydroxy-diphenylene sulfone. We have no evidence as to the position of the hydroxyl group in the benzene ring.

DISCUSSION

The close chemical relationship of diphenylene sulfide to diphenylene oxide invites comparison of the effects produced by continued feeding of the compounds. The present investigation has shown that diphenylene sulfide at a level of .05 per cent of the diet inhibits the growth rate of albino rats, an effect attributed to the decreased food intake. At a concentration of .10 per cent inhibition of growth is still chiefly due to inanition. In sharp contrast to these findings the earlier work by Thomas, Wilson, and Eddy (1940) on diphenylene oxide showed that no decrease in food intake,

nor inhibition of growth, occurred on a dosage level as high as .40 per cent of the diet.

Neither diphenylene sulfide nor diphenylene oxide produced any change in the blood picture. Both compounds caused the appearance of fine brown-pigment granules in the epithelium of the proximal convoluted tubules of the kidneys, but without evidence of degeneration of the epithelial cells.

Diphenylene sulfide produced enlargement of the liver on the higher dosage levels, and, as judged by histological examination, fatty degeneration of the liver on all dosage levels. Feeding of diphenylene oxide, even in much higher dosages, produced no such effects.

These two compounds exhibited one other interesting difference when fed to albino rats over long periods of time. Diphenylene sulfide had no effect on water intake and urine output, whereas the oxide caused an increased water intake and urine excretion.

The dissimilar toxicities of these two closely related compounds again illustrate the general thesis that toxicity data on one compound do not necessarily justify conclusions as to the probable toxicity of some other closely related compound.

The amounts of diphenylene sulfide ingested per rat during the 165-day period were, for the three concentrations, respectively: .025 per cent, .481 gram; .05 per cent, .889 gram; and .10 per cent, 1.496 grams. Since fatty degeneration of the liver was found in the rats receiving the lowest dosage, we may, for purposes of transferring the data to man, base our calculations on the lowest level of diphenylene sulfide consumed. The average weight of the rats receiving the .025 per cent diphenylene sulfide diet was approximately 200 grams for the 165-day period, and the total amount of diphenylene sulfide ingested was .481 gram per rat. This is equivalent to .0145 gram per kilo of body weight per day. If the average weight of a man is assumed to be 60 kilos, the data on rats would indicate that the daily ingestion of .87 gram of diphenylene sulfide would produce liver injury. This conclusion assumes that rats and human beings are equally susceptible to the toxic action. It must be remembered that considerable liver injury was produced in rats receiving a diet containing .025 per cent diphenylene sulfide, but we do not know how small a dosage would continue to produce fatty degeneration of the liver. In order to have an adequate factor of safety in passing judgment for the presence or absence of a public-health hazard it is therefore necessary to assume that liver damage might be produced in man by quantities of diphenylene sulfide smaller than .0145 gram per kilogram of body weight per day. Certainly the probability of public health hazards traceable to spray residues of diphenylene sulfide would seem to be much greater than in the case of diphenylene oxide.

SUMMARY

The continued feeding of diphenylene sulfide to young male albino rats for 165 days resulted in lessened growth rates on concentrations of .10 and .05 per cent, but not on .025 per cent, in the diet.

Food consumption decreased as the slopes of the growth curves decreased, and varied inversely with the concentration of diphenylene sulfide

in the diet. Retardation of growth on the .05 per cent dosage level was due to the decrease in food intake. Even on the diet containing .10 per cent diphenylene sulfide inanition was chiefly responsible for the inhibition of growth.

The spleens of the experimental animals decreased in weight as the dose of the sulfide increased. The histology of the organ, however, remained normal.

A light brown pigmentation of the epithelial cells of the proximal convoluted tubules of the kidneys was seen. There was no evidence of cell destruction.

There was no evidence of blood injury.

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VITAMIN CONTENT OF GREEN SNAP BEANS. INFLUENCE OF FREEZING, CANNING, AND DEHYDRATION ON THE CONTENT OF THIAMIN, RIBOFLAVIN, AND ASCORBIC ACID[1,2]

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Surprisingly little study has been given to the vitamin content of fresh, frozen, canned, and dehydrated snap beans. In 100 grams of green beans, Miller (1927) found 60γ of thiamin; Quinn, Burtis, and Milner (1927) found 45γ; Rogers (1928) an average of 54γ; and Booher and Hartzler (1939) 72γ. Mayfield and Richardson (1939) reported that snap beans in the fresh, raw state are very good sources of vitamins B_1 and C. They showed that a 30 to 40 per cent loss of vitamin C and no loss of vitamin B_1 occurred when the vegetable was boiled for 45 minutes. When canned snap beans were stored for eight months, losses of 80 per cent and 40 per cent were noted for vitamins C and B_1, respectively. Sherman and Burton (1926) reported that the rate of destruction of vitamin B_1 was increased when the pH value of the cooking medium was increased from 4.2 to 5.2. The rat-bioassay method was used for vitamin B_1 by all the above-mentioned investigators.

That the vitamin C content is variable is shown by reference to the values tabulated from several reports by Daniel and Munsell (1937). Mack, Tapley, and King (1939) reported that little ascorbic acid was destroyed by boiling, but that 33 per cent was extracted into the cooking water. They also found that while the raw beans lost ascorbic acid at all temperatures during storage, the losses were much greater and more rapid at higher temperatures.

No data showing the effect of various processing methods on the riboflavin content of green snap beans has come to our attention.

This paper reports results on fresh, frozen, canned, and dehydrated green snap beans *as purchased* and not *as served*. However, in order that the present data may be interpreted on the *as served* basis, we have determined the actual vitamin losses involved in cooking representative samples of these products (Table 4). Since none of the fresh, raw beans was available, the cooking losses were determined on the Bountiful variety of green snap beans from another lot grown the following season.

EXPERIMENTAL PROCEDURE

Bountiful variety of green snap beans of the same degree of maturity when picked were used. The beans were grown on the plots of the Massachusetts Agricultural Experiment Station at Amherst, Massachusetts. Only

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[2] Presented before the Agricultural and Food Division, American Chemical Society, at the 101st Meeting, St. Louis, Mo., April 9, 1941.

young beans from 15 to 20 days old (from time of blossoming) were used, because Culpepper (1936) has shown that the composition of the entire snap bean varies greatly at different stages of development as a result of changes going on in its two parts—the pod valves and the seeds.

The beans used for freezing were sorted and washed previous to blanching for two minutes in boiling water. The blanched beans were dipped in cold water, drained, and immediately packed without salt or brine in one-pint, E-Z Seal, all-glass jars. The jars were sealed but not exhausted for air removal. Freezing took place in 65 to 75 minutes at —4° F.(—20°C.).

TABLE 1

Effect of Blanching, Freezing, Canning, and Dehydration on Thiamin Content of Bountiful Variety Green Snap Beans

Product	Moisture content	Thiamin content per 100 grams				
		Freshly processed		Loss	After storage for one year	
		Wet basis	Dry basis		Dry basis	Percentage loss[1]
	pct.	*micrograms*	*micrograms*	*pct.*	*micrograms*	*pct.*
Raw, fresh beans[2]	90.6	63	669	....	....	....
Blanched and frozen in glass container	92.3	77	996	....[3]	780	22
Unblanched and frozen in glass container	90.6	57	612	39.0	261	74
Blanched, canned in tin can	93.2	54	825	17.0	558	44
Blanched, canned in glass container; pH 5.3	93.2	54	822	17.0	549	45
Blanched, acidified, canned in glass; pH 4.2	93.2	60	897	10.0	627	35
Blanched, canned in glass, head space 1½ in.	93.2	54	819	18.0	618	38
Blanched, dehydrated, stored in glass	2.9	....	981	1.5	837	16

[1] Frozen samples stored at —4°F.(—20°C.); all other samples stored at 38°F.(3.3°C.).
[2] Picked fresh every six days, stored at 38°F.(3.3°C.). [3] Used as basis for calculating losses.

The frozen beans were held at this temperature until assayed. One jar was used for two feedings. The frozen beans were finely ground in a newly galvanized vegetable chopper previous to feeding each day. Defrosted beans were never used.

A second portion of raw, fresh beans was frozen without blanching.

A third portion of the snap beans was blanched for two minutes in boiling water, drained, and dehydrated at 130 to 145°F.(54.4 to 62.8°C.) for 36 hours in a tunnel-type, hot-air drier. Near the end of the dehydration period the temperature reached 150°F.(65.7°C.) for a short time. The dehydrated beans were stored in the dark in sealed, but not vacuumized, glass containers. The moisture content was 2.9 per cent.

A fourth portion of the snap beans was canned. The preliminary treatment was the same as for freezing. No. 2 plain tin cans and E-Z Seal, glass, pint jars were used. Exactly 250 grams of blanched beans and 200 grams of

water at 175°F.(79.4°C.) were used in each glass jar, while 275 grams of beans and 210 grams of hot water were filled into each tin can. The head space in the glass jars was nearly twice that of the cans. Processing was at 240°F.(115.6°C.) for 20 minutes in a temperature-controlled retort. Both types of containers were water-cooled after processing. Storage of the canned beans was at 38°F.(3.3°C.) in a dark room.

Fresh beans were harvested every week during the assay period and were stored at 38°F.(3.3°C.) until used. The solid portion and the free-run

TABLE 2

Effect of Blanching, Freezing, Canning, and Dehydration on Vitamin B_2 or Riboflavin Content of Bountiful Variety Green Snap Beans

Product	Moisture content	Riboflavin content per 100 grams				
		Freshly processed		Loss	After storage for one year	
		Wet basis	Dry basis		Dry basis	Percentage loss[1]
	pct.	*micrograms*	*micrograms*	*pct.*	*micrograms*	*pct.*
Raw, fresh beans[2]	90.6	132	1,404	....	....	....
Blanched and frozen in glass container	92.3	156	2,007	....[3]	1,947	3.0
Unblanched and frozen in glass container	90.6	144	1,530	24	1,230	39.0
Blanched, canned in tin can	93.2	132	1,940	3	1,821	9.0
Blanched, glass-packed; pH 5.3	95.7	84	1,953	2	1,857	7.5
Blanched, glass-packed, acidified; pH 4.2	95.7	84	1,953	2	1,941	3.0
Blanched, glass-packed	95.6	87	1,971	2	1,854	8.0
Blanched, dehydrated, stored in glass	2.9	....	2,019	0	2,004	0

[1] Frozen samples stored at —4°F.(—20°C.); all other samples stored at 38°F.(3.3°C.).
[2] Picked fresh every six days, stored at 38°F.(3.3°C.). [3] Used as basis for calculating losses.

juice of the canned samples were assayed in order to determine what percentage of these vitamins went into solution in the juice.

The standard method of vitamin B_1 rat assay, as described by Booher and Hartzler (1939), was followed. The results were read from reference growth curves obtained by feeding graded amounts of pure thiamin chloride to rats. The vitamin B_2 (G) content of the samples was determined by the rat-growth method of Bourquin and Sherman (1931). The results are expressed in microgram (gamma) equivalents of riboflavin by means of reference growth curves obtained by feeding graded doses of riboflavin. Three feeding levels were used for both thiamin and riboflavin. The Tillmans method (1932) as modified by Bessey and King (1933) and by Mack and Tressler (1937) was used to determine the vitamin C content of the products and the results are expressed as milligrams of ascorbic acid per gram. Moisture determinations were made on all the products and the results are reported on a moisture-free basis except in the case of the juices. The data

showing the effect of various methods of processing on the vitamin content of green snap beans are presented (Tables 1, 2, and 3).

DISCUSSION OF RESULTS

Since the vitamin B_1 and B_2 values for blanched and quick-frozen, snap green beans were considerably higher than were the values found for fresh snap beans, processing losses are based on the maximum values of the blanched, frozen beans. It seems likely that the thiamin content of the fresh beans decreased as the season advanced. There may also have been a loss owing to storage of freshly picked samples for one to five days before feeding the rats. Again, it is possible that the thiamin in the raw beans is not fully utilized by the rat. In snap beans that were quick-frozen but not blanched, there was a 39-per cent loss of vitamin B_1; in one year the loss increased to 74 per cent. These losses are probably due to enzyme action. Of course, commercial frozen foods are always blanched in order to inactivate enzymes. Apparently, temperatures as low as —4°F.(—20°C.) are not sufficient to inactivate the enzymes.

There appeared to be little loss of vitamin B_1 in snap beans that were blanched and then dehydrated in a tunnel-type, hot-air dehydrator. At the normal pH of 5.5, canning resulted in a 17-per cent loss, and when the pH was decreased to 4.3 with citric acid, the loss was decreased to 10 per cent. The type of container, whether tin or glass, had no effect on the vitamin retention. Blanched, quick-frozen snap beans lost a total of 22 per cent of

TABLE 3

Effect of Blanching, Freezing, Canning, and Dehydration on Vitamin C (Ascorbic Acid) Content of Bountiful Variety Green Snap Beans

Product	Moisture content	Ascorbic acid per 100 grams [1]				
		Freshly processed		Loss	After storage for one year	
		Wet basis	Dry basis		Dry basis	Percentage loss [1]
	pct.	*micrograms*	*micrograms*	*pct.*	*micrograms*	*pct.*
Raw, fresh beans [2]	90.6	26.5	283	....	....	....
Blanched, frozen in glass container	92.3	14.5	188	33	150	47.0
Unblanched, frozen in glass container	90.6	6.5	70	75	27	90.5
Blanched, canned in tin can	93.2	4.5	71	75	68	76.0
Blanched, glass-packed; pH 5.3	93.2	4.3	63	78	60	79.0
Blanched, glass-packed, acidified to pH 4.2	93.2	5.0	74	74	71	75.0
Blanched, glass-packed, head space 1½ in.	93.2	4.0	59	79	57	80.0
Blanched, dehydrated, stored in glass	2.9	....	15	95	12	96.0

[1] Frozen samples stored at —4°F.(—20°C.); all other samples stored at 38°F.(3.3°C.).
[2] Picked and used the same day for ascorbic acid determinations; content of ascorbic acid in fresh beans used as basis for calculating losses.

their thiamin content in 12 months' storage at —4°F.(—20°C.), whereas canned snap beans stored at the same temperature lost 45 per cent. Dehydrated snap beans lost but 16 per cent and the unblanched, quick-frozen beans lost one-half of their original thiamin content in storage at —4°F. (—20°C.), making a net total loss of 75 per cent.

It is clear that there was no significant loss of vitamin B_2 (less than 10 per cent) in the quick-freezing, canning, or dehydration processes; nor was there any appreciable loss after storage for 12 months (Table 2). As in the case of vitamin B_1, the lower value of vitamin B_1 for the fresh product may be attributed to enzymic action or possibly lessened digestibility. In other words, vitamin B_2 is remarkably stable to the operations incidental to freezing, canning, and drying.

A more pronounced effect of quick-freezing and canning on the ascorbic acid content of green snap beans is readily noticed (Table 3). Quick-frozen, blanched snap beans lost 33 per cent of their vitamin C, whereas the canned products lost approximately 75 per cent. The latter value is in close agreement with that of Mayfield and Richardson (1939). Omission of the blanching process accounts for a 75 per cent destruction of vitamin C in frozen beans, whereas the blanched beans lost only 33 per cent. Retention of vitamin C was the same in glass and in tin containers. Acidification of the beans was of doubtful value except possibly in the case of thiamin retention. Twelve months' storage had no effect on the ascorbic acid content of the canned snap beans, but the blanched, frozen snap beans lost an additional 14 per cent and the unblanched ones retained only 10 per cent of their original content of ascorbic acid. Presumably, this loss can be attributed to the action of ascorbic acid oxidase. Dehydration of the product resulted in almost total destruction of ascorbic acid.

VITAMINS IN LIQUID PORTIONS OF CANNED SNAP BEANS

In preparation of the glass-packed beans, 250 grams of blanched beans and 200 grams of water were added to each jar. Vitamin determinations were made separately on the solid and liquid contents of the jars with the following results:

Thiamin present in solid portion	54 γ per 100 grams
Thiamin present in liquid portion	21 γ per 100 grams
Percentage of thiamin in liquid portion	30.3 per cent
Riboflavin present in solid portion	132 γ per 100 grams
Riboflavin present in liquid portion	36 γ per 100 grams
Percentage of riboflavin in liquid portion	27.6 per cent
Ascorbic acid present in solid portion	4.5 mg. per 100 grams
Ascorbic acid present in liquid portion	4.5 mg. per 100 grams
Percentage of ascorbic acid present in liquid portion	50 per cent

The above data show that the liquid contents of canned snap beans contain relatively large percentages of the vitamins. In four separate tests for vitamin C in tin cans and in glass jars, the average percentage present in the liquid 30 days after canning was almost exactly 50 per cent. About 30 per cent of the thiamin and riboflavin in the canned beans was found in the liquid portion. It is clear that the liquid portion of canned vegetables should be utilized in order to obtain the full nutritive value of the food.

After the foregoing data had been accumulated, it was suggested that the results would be of greater value if the vitamin losses could be compared on the several products after cooking on the *as served* basis. Consequently, samples of the frozen, canned, and dehydrated beans were cooked, and vitamin determinations were made before and after cooking. The samples were 16 months old when the cooking tests were made. The thiamin was determined by the Melnick and Field (1939) procedure and the riboflavin by means of the microbiological method of Snell and Strong (1939). Duplicate determinations were made on the samples. Freshly picked, Bountiful variety, snap beans from a source totally different from that for the series reported here, were used to ascertain the losses owing to cooking in the fresh beans.

TABLE 4

Losses in Vitamins Owing to Cooking Green Snap Beans, Bountiful Variety[1]

Product	Ratio of beans to water by weight	Exposed to heat, total	Vitamin losses		
			Vitamin C	Vitamin B_1	Riboflavin
		min.	*pct.*	*pct.*	*pct.*
Fresh beans	4:1	30	24	10	0
Frozen beans	4:1	16	12	0	0
Canned in glass	1:0	6	6	0	0
Canned in tin	1:0	6	5	0	0
Dehydrated	(Soaked 24 hrs.) 1:10 (Cooked)				
	4:1	40	....[2]	45	33

[1] Total vitamins present in solid and liquid portions. [2] Too small an amount was present in dehydrated beans to allow for an accurate estimate of loss owing to cooking.

The results are self-explanatory but show very small or no losses in thiamin and riboflavin owing to cooking. The dehydrated beans constitute an exception. A little thiamin seems to be lost in cooking the fresh beans. The greatest loss in ascorbic acid content was in cooking the fresh beans; this was followed by the frozen and canned samples. It must be kept in mind that the accuracy of the methods used probably does not give significance to values less than 10 per cent. In the soaking process incidental to cooking the dehydrated beans, there are marked losses of both thiamin and riboflavin. The ascorbic acid was largely destroyed during dehydration and storage so that little remained at the time of soaking in water. It seems, therefore, that conclusions reached on the basis of snap beans *as purchased* are valid also for the most part for the beans *as served* after cooking.

SUMMARY

The thiamin, riboflavin, and ascorbic acid values of frozen, canned, and dehydrated snap beans of the Bountiful variety were determined.

Quick-freezing, when preceded by a two-minute blanching (scalding) period, conserved vitamins B_1, B_2 (G), and C in snap beans to the highest degree of all processing methods. Moderate losses of vitamin B_1 occurred in canning but the canned products lost about 45 per cent after storage for

12 months. Vitamin B_2 (G) appeared to be stable to freezing, canning, storage, and dehydration. It is apparently destroyed to some degree by enzyme action.

Blanching was definitely proved to be of utmost importance in the conservation of the vitamins.

Dehydrated beans lost little of their vitamins B_1 and B_2 even on long storage. Vitamin C was almost totally destroyed by dehydration.

About 30 per cent of the thiamin and riboflavin was present in the liquid portion of the canned product. Approximately 50 per cent of the ascorbic acid was found in the liquid contents.

Vitamin C appeared stable to long storage after canning in glass or tin containers.

In conclusion, it may be stated that while quick-frozen snap beans retain considerably higher percentages of vitamins B_1 and C (and equal values of vitamin B_2) than canned, both quick-frozen and canned snap beans are good sources of these three water-soluble vitamins. Tin and glass containers are equally satisfactory in retaining these vitamins.

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BACTERIOLOGICAL SURVEY OF CANNED SALMON

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It has been almost 20 years since a bacteriological survey of canned salmon was made, and during this time there have been a number of changes in packing procedure. Certain of these changes, notably the use of a mechanical vacuum in place of the steam exhaust, are of possible significance with respect to sterility of the finished product. Canned salmon is given a severe process—90 minutes at 116.6°C.(242°F.)—in order to soften the bones and make them edible. This process is in excess of the cook necessary to protect against spoilage as calculated from heat-penetration data. Consequently no reports of spoilage owing to understerilization have been made. However it was felt that experience with other products, where a change in packing procedure had introduced possibilities of spoilage, justified and made desirable a bacteriological survey of canned salmon. Furthermore, cultures of canned salmon without controls occasionally showed one or a few colonies of non-heat-resistant bacteria. These were considered to be culture contaminants, but no information on the extent or significance of such air-borne contamination was available, and this point seemed of sufficient importance to merit investigation. Since the work was completed there has appeared an excellent paper by Mudge and Foord (1940) in which attention is called to the problem of air-borne contamination and to the use of multiple controls to indicate the extent of such contamination.

The present report is based upon the examination of 111 cans of salmon, including four half-pound cans of Columbia River Chinook, with added salmon oil, and 107 one-pound cans of Alaska salmon distributed as follows: pink, 32 cans; chum, 24 cans; red, 27 cans; and king, 24 cans. One can of pink salmon and two cans of red carried distributors' labels. The remaining lot of 108 cans, representing 12 packers and 13 canneries, was in part purchased on the open market and in part selected at random from warehouse stock. These cans may be considered as fully representative of the bacteriological condition of commercial canned salmon.

REVIEW OF LITERATURE

Most of those who have reported on the bacteriological examination of canned salmon have stated that bacteria could be recovered from an appreciable percentage of normal cans. The only exception appears to be Bushnell and Utt (1917), who recorded negative results on 52 cans purchased on the open market and representing 26 manufacturers. Weinzirl (1919) found two cans among 17 cultured to show bacteria, but since these occurred among cans with imperfect seams, this observation loses in significance as an index

to the effectiveness of the process. Cheyney (1919) incubated 34 cans at 37°C.(98.6°F.) for 10 days and recovered bacteria from seven (20 per cent) of these. He stated that the cans represented processes ranging from 30 minutes at 115.5°C.(240°F.) to 85 minutes at 117.7°C.(244°F.). It is unlikely that many of the cans were from lots given the lower processes else a higher percentage of non-sterile cans would have been noted, since Fellers (1926) states that the center temperature of a pound can is only 100°C. (212°F.) after 30 minutes at 115.5°C. Hunter and Thom (1919) found 44.7 per cent of 530 cans cultured to contain bacteria, and DeBord (1920) reported 34 per cent non-sterile cans among 1,283 examined bacteriologically, but in neither instance are the results likely to be fully representative of average commercial quality since the cans were apparently from lots under restraint. Savage, Hunwicke, and Calder (1922) state that eight of 16 normal cans purchased on the open market were non-sterile. They noted cocci in four instances and a nonsporeforming rod in one instance, and discussed at some length from a theoretical standpoint the conditions necessary for spoilage to occur in canned food which is not bacteriologically sterile in sound cans. In commenting on the presence of micrococci it was stated, "Their heat resistance when surrounded by meat or fish tissue is probably a very different thing from that exhibited in broth cultures." Apparently no tests of heat resistance in tissue were made. Fellers (1926) has reported the results of the examination of 7,664 cans drawn from 540 lots, of which 324 were of normal quality and 216 of doubtful quality. The normal quality lots included 5,276 cans and of these 3.4 per cent were non-sterile. The lots of doubtful quality showed 7.1 per cent non-sterile among 2,398 cans. The types of bacteria found in 220 of the positive cans included aerobic sporeforming rods, facultative anaerobes, obligate anaerobes, cocci (16 times), nonsporeforming rods (16 times) and yeasts and molds (one time). It is of some interest to note that bacteria were numerous in only two lots and that these were actually undergoing spoilage. He found it difficult to account for the presence of cocci and nonsporeforming rods in normal cans and assumed that there was some protective agency in salmon which permitted such types to survive the process. Another puzzling observation was that sporulating aerobes from normal cans would grow upon reinoculation into salmon and would cause softening of the tissue, abnormal odor, and discoloration.

A highly probable explanation of the results of Savage *et al.*, and of certain of those of Fellers, and one which requires no theoretical reasoning or assumption, is to be found in the work of Mudge and Foord (1940). It is only necessary to recognize the fact that despite the exercise of the greatest possible care air-borne contamination can occur. Mudge and Foord state that non-sterile controls were observed even when the plates were poured in a case to which access was possible only through small armholes. Roe (1940) has noted that plates carefully poured, dried with porcelain lids, and inoculated with sterile broth showed contaminants on as high as 15 to 20 per cent when the operations were carried on in the laboratory atmosphere. Discussion with several other bacteriologists and our own experience support

the observations of these workers and emphasize the importance of multiple controls in tests for absolute sterility.

EXPERIMENTAL PROCEDURE

Sampling and Cultural Procedure: At the time of culturing, each can was wiped with a damp cloth to remove dust particles and one end thoroughly flamed by holding inverted over a burner. This resulted in slight pressure in the can and prevented sucking in of air when opened. The flamed end was opened with a flamed spiral opener. The can was kept adjacent to a burner during all culturing operations with the idea that the rising currents of air would minimize the settling out of air-borne dust particles. Liquid was removed with a large-bore pipette and distributed among Petri dishes and culture tubes, except in those instances where oil from the can was to be emulsified. In these cases the liquid and oil were poured into a large test tube, the oil allowed to rise, then pipetted off for emulsification, and the liquid remaining in the tube was cultured. Tissue for culturing was removed from the first 25 cans with sterile spoons. This method of sampling proved to be somewhat clumsy and the rest of the cans were sampled with a cork borer. Between the sampling of successive cans, the cork borer was held for several minutes in boiling Oakite solution to remove the oil and tissue and was then immersed in alcohol. Just before use the alcohol was burned off and the borer was well flamed. It was necessary to handle it with crucible tongs but this was not a handicap. The tissue sample was forced from the borer with a pipette. Recently our attention has been called to the use of a cheese trier as a sampling device and there seems no doubt that this instrument is superior to the cork borer.

The amount of liquid added to each Petri dish was approximately three ml. Two plates were incubated at 37°C. and two at room temperature. The amount of tissue removed with each sampling was of necessity slightly variable but it is believed that it would average about 25 grams. For plate cultures the tissue sample was added to 20 ml. of sterile water, thoroughly broken up with a pipette, and the coarse particles allowed to settle out after which the supernatant liquid was distributed in three-ml. quantities to each of the four Petri dishes—two for incubation at 37°C. and two for incubation at room temperature. Oil from 18 cans was cultured according to the following technic. Three ml. of oil were added to 15 ml. of a sterile solution of gum tragacanth and gum arabic (about two and one per cent, respectively). After emulsification was effected three ml. were added to each of four plates for incubation in duplicate at 37°C. and room temperature.

Tube cultures included two tubes each for aerobic and anaerobic culture of both liquid and tissue. Each of the liquid tubes received three ml. of inoculum, and each of the tissue tubes received approximately 25 grams of tissue which was thoroughly broken up with a pipette. One tube of each set was incubated at 37°C. and one at room temperature. Cultures at 37°C. were incubated for three days and those at room temperature for five days before observation.

The plating medium was Bacto-dextrose-tryptone agar. The liquid media included Dextrose-tryptone broth for aerobic culture, and liver broth with

liver tissue stratified with nutrient agar after inoculation for anaerobes. Tubed media were in 8 x 1-inch test tubes, each containing about 30 ml. of medium.

All media and glassware were freshly sterilized. All work was done in a small inoculating room ventilated with filtered air. Two technicians coöperated on the culturing operations in order to shorten the period of exposure of the opened can to the air. As noted earlier the opened can was kept adjacent to a burner to minimize air-borne contamination. As an additional check on air contamination, two open Petri dishes of solidified plating medium were held immediately adjacent to the can throughout the sampling. As a control on the pouring procedure two sterile, uninoculated dishes were poured at the same time as the inoculated plates. It should be emphasized that for these controls the lid of the dish was lifted just enough

TABLE 1

Percentage of Petri Dishes Showing Growth

Inoculum	Liquid		Tissue		Oil		Controls			
							Exposure plates		Pouring controls	
Temperature of incubation	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.
Positive for bacteria only	32	32	34	28	33	25	85	71	25	30
Positive for bacteria and molds	1	3	1	3	0	0	10	22	3	6
Positive for molds only	4	10	1	9	6	12	5	7	2	4
Total positive all types of growth	37	45	36	40	39	37	100	100	30	40

to permit pouring, and hence exposure to contamination was as slight as possible. One each of the exposure control and the pouring control plates was incubated at the two temperatures along with the inoculated plates.

Smears were made from the first 25 cans cultured but microscopic examination of these was unsatisfactory because of artifacts, and consequently no microscopic examination was attempted on the remaining cans.

Liquid from the cans was used for pH determinations with a Beckman glass electrode potentiometer. Organoleptic examination was made of each can.

Results: Qualitative cultural results, so far as plate cultures are concerned, are given in summary form (Table 1), in which the percentage figures have been given to the nearest whole number. Detailed quantitative results on 16 cans including four each of the Alaska varieties are shown (Table 2). This abridged table is fully representative since no significant difference may be found between individual cans.

In general, it may be said that with the exception of the exposure controls those plate cultures which were positive for bacteria showed chiefly one to three colonies per plate. These were almost always surface colonies

TABLE 2

Detailed Cultural Results From Representative Cans

Packer No.	Variety	pH	Plate cultures				Tube cultures—aerobic				Tube cultures—anaerobic				Control plates			
			Liquid		Tissue		Liquid		Tissue		Liquid		Tissue		Exposure		Poured	
			37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.	37°C.	R.T.
1	Pink	6.10	0-1b[1]	0-0	0-1b	0-0	—	—	—	—	—	—	—	—	Spr.	7b	0	1b
2	Pink	6.15	0-0	Spr.-m	0-1b	Spr.-3m	—	—	—	—	—	—	—	—	3b	16b,2m	0	8b,1m
3	Pink	6.16	0-spr.	0-0	0-0	1m-2b,1m	—	+	—	—	—	—	—	—	Spr.	7b	0	0
4	Pink	6.18	0-0	1b-1b	0-0	1b-1m	—	—	—	—	—	—	—	—	4b	5b,2m	0	1b
5	Chum	6.10	0-1m	0 0	0-1b	0-0	—	—	—	—	—	—	—	—	Spr.	Spr.	0	0
6	Chum	6.09	3b-0	0-0	2b-spr.	0-0	—	—	—	—	—	—	—	—	8b,1m	4b	0	0
3	Chum	6.14	Spr.-1b	Spr.-x	0-spr.	3b-0	+	—	—	—	—	—	—	—	8b,1m	8b	2b	1b
1	Chum	6.07	3b-0	0-spr.	0-0	0-0	—	—	—	—	—	—	—	—	11b	24b	0	0
7	King	6.19	0-0	0-0	0-0	0-0	—	—	—	—	—	—	—	—	6b,1m	7b,1m	1b,1m	0
8	King	6.22	1b-1b	0-0	0-0	0-2b,1m	—	—	—	—	—	—	—	—	11b	8b	1b	1b
9	King	6.10	0-0	0-0	0-0	0-0	—	—	—	—	—	—	—	—	2b	3b	0	1b
6	King	6.28	0-1b	0-1m	0-0	0-0	—	—	—	—	—	—	—	—	4b	3b	0	0
10	Red	6.21	0-0	1m-2b,1m	2b-0	0-0	—	—	—	—	—	—	—	—	Spr.	Spr.	0	2b
3	Red	6.15	0-0	Spr.-1b	0-0	1b-x	—	—	—	—	—	—	—	—	m	m	1b	0
9	Red	6.13	0-0	0-0	0-0	0-0	—	—	—	—	—	—	—	—	3b	6b	0	0
8	Red	6.20	0-0	0-0	1b-2b	0-0	—	—	—	—	—	—	—	—	6b	4b	1b	0

[1] Code: 0 = no growth; spr. = spreader; x = plate broken; b = bacterial colony; m = mold colony.

and where more than one colony was present they were usually of dissimilar types. They included aerobic sporeformers of the subtilis group and both pigmented and nonpigmented cocci. A fairly large percentage of the positive plates showed the presence of mold colonies also.

Any significance which the results on the inoculated plates might be considered to have suffers when the control plates are considered. As might be expected the exposure controls showed the heaviest growth, all being positive and with colony counts as high as 24 being recorded. Although the opportunity for contamination was much greater with the inoculated plates than in the instance of the controls on pouring, neither the percentage of plates showing growth nor the number of colonies per plate is appreciably greater. Counts on these controls were usually one per plate but as high as eight was recorded, and in several cases subsurface growth was noted. The types of bacteria on the control plates were similar to those found on the inoculated plates. The only difference was in amount of growth—the exposure plates showing the heaviest seeding, the inoculated plates next, and the control on pouring least. Referring to the 72 plates inoculated with emulsified oil, only 27 showed growth and of these only five had more than one colony per plate, with a maximum count of three. In only two instances were duplicate plates positive and with these it was obvious that contamination from outside had taken place.

The positive tubes were all isolated instances showing no correlation with the plate results. With the two inocula and two temperatures of incubation, aerobic growth was obtained in 21 and anaerobic growth in two of 444 inoculated tubes each, corresponding to five per cent and .5 per cent, respectively. The organisms were similar to those isolated from plates.

The pH values ranged from 5.94 to 6.39 with the great majority of the determinations being in the range of 6.1 to 6.2. These values are considered to have been normal.

Organoleptic examination revealed no abnormalities in appearance, flavor, or odor.

GENERAL COMMENT

In any bacteriological operation involving cultures or nonselective media for incubation at temperatures favorable to air-borne types, unusual precautions are necessary both in the handling of the material and in the interpretation of the results. Otherwise, the true results will become obscured because of the presence of nonsignificant forms. Under some conditions air-borne contamination is unlikely to constitute a serious problem. Thus no problem is presented where a selective medium which shows a characteristic growth, such as the bismuth sulfite medium for the isolation of *Eberthella typhosa*, or a selective temperature of incubation, as used in the cultivation of thermophilic types, serves to eliminate from consideration contaminating forms. Numerous other examples could be cited. However, in testing for sterility any material, and especially one where cultures are made on ordinary nutrient medium and incubated at mesophilic temperature, air-borne contamination may be very confusing. It is unlikely to amount to more than a few colonies on any plate and, as Mudge and Foord state, "normally, the bacteriologist disregards such colonies." Where the

problem involves the determination of absolute sterility there has always been the question of whether he is justified in disregarding the presence of even a single colony on the test plates. According to our experience about one out of three to four uninoculated plates will show bacterial growth of one type or another when the pouring is done under the conditions described above. If only a single control plate is poured as a test of sterility of media and glassware, and if this should remain sterile while one of each two to four plates of a test series shows growth, then an entirely erroneous idea of the bacterial condition of the material tested may develop. Clearly a greater number of controls would be of help in the interpretation of such results.

In any sampling and culturing operation according to the procedures commonly used in bacteriological laboratories there is a greater chance of air-borne contamination on test plates than on control plates. The various manipulations all afford an opportunity for contamination so that both the percentage of non-sterile plates made from a truly bacteriologically sterile material, and the average number of colonies per plate, should be higher among the inoculated plates than among the controls. We do not have sufficient information to justify a statement as to the range of difference, but we feel that no exception can be taken to a demand that there be a substantially higher number of positive plates among the test series than among the control series before a statement imputing lack of sterility to a material is justified. With a material of such excellent nutritive property as salmon neither the differences in percentage of plates showing growth (Table 1) nor the quantitative results (Table 2) can be considered as significant in the absence of organoleptic evidence of non-sterility. The fact that the bacterial flora of all plates, both inoculated and control, showing growth was similar in all respects except amount, and also that this flora was the typical air-borne type indicates that the origin of the organisms was in fact the air.

SUMMARY

A general consideration of all results, cultural, pH, and organoleptic, leads to the conclusion that all cans tested were bacteriologically sterile. It may be inferred therefore that changes in packing procedure have introduced no new spoilage hazard, and that the process in use is adequate to ensure sterilization. Attention is called to the importance of multiple controls in cultural tests for absolute sterility.

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DETECTION OF *BACILLUS THERMOACIDURANS* (BERRY) IN TOMATO JUICE, AND SUCCESSFUL CULTIVATION OF THE ORGANISM IN THE LABORATORY

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During the first few years of manufacture of tomato juice there was a number of outbreaks of what came to be known as "flat-sour" spoilage, characterized by the development in varying degree of intensity of a medicinal or "phenolic" taste, and accompanied by a drop of some .3 to .5 in pH. Cans of spoiled juice were observed to remain flat, but there was always a loss of vacuum. Since empirical methods for its control have been developed, this type of spoilage at the present time is of rare occurrence.

Microscopic examination of spoiled juice always revealed the presence of an abundance of large, rod-shaped, vegetative cells, but only rarely was subsequent subculturing successful. Occasionally isolations of aerobic, mesophilic, spore-forming bacteria could be made, but these failed to grow when reinoculated into juice and consequently were considered to be non-significant survivors of the process. Berry (1933) was successful in isolating a spore-forming organism which he described under the name *Bacillus thermoacidurans*, and which would reproduce the characteristic picture upon reinoculation, but very few isolations have been made by other investigators. Even the organism isolated by Berry was of uncertain and irregular behavior in experimental work with tomato juice. Apparently there was an attenuation, or complete loss, of ability to grow in juice. Furthermore sporulation was scanty so that experimental work with spores was difficult or impossible.

The investigations reported in this paper were undertaken in an effort to overcome some of the difficulties which hampered work on flat-sour spoilage of tomato juice. The culture studied was a transplant of the original Berry isolation and was obtained from the American Can Company.

EXPERIMENTAL PROCEDURE

Acclimatization of the Culture: A large number of tubes of tomato juice and of a medium consisting of equal parts of filtered tomato juice and dextrose tryptone broth were inoculated with the original culture and incubated at 37°C.(98.6°F). for several weeks with frequent observation for growth but with negative results. When the experiment was repeated, however, growth took place in one tube of juice and transplants from this tube to other tubes of juice yielded some positive cultures. Repeated transplanting from juice to juice eventually developed a culture which would always cause spoilage. All work has been done with this acclimatized strain.

Development of a Culture Medium: Since *B. thermoacidurans* was found to grow poorly on most media employed in the laboratory, it was necessary to develop a medium which would support abundant growth of the organism

and at the same time be easily prepared. Various combinations of ingredients were tested in an effort to obtain a satisfactory substrate. These included peptone, tryptone, proteose peptone, yeast extract, beef extract, tomato juice, and glucose. Sodium acetate and potassium phosphate were used as buffers, and citric and hydrochloric acids as acidifying agents. The basis of comparison included luxuriance of growth and quantitative counts of a suspension of a pure culture. The optimum pH was determined by comparing growth on the same medium at several different pH values. The composition of the medium that proved most satisfactory is given below.

Proteose peptone (Difco)	5 gm.
Yeast extract (Difco)	5 gm.
Glucose	5 gm.
K_2HPO_4	4 gm.
Distilled water	500 ml.

The ingredients are dissolved in the water, and the pH adjusted to 5 with dilute HCl. In a separate flask 20 grams of agar are dissolved in 500 ml. of water, and then both solutions are sterilized for 30 minutes at 15 pounds pressure. When the medium is ready to be used, the melted agar is mixed with the sterilized nutrients at 45 to 55°C.(113 to 131°F.).

This medium, designated as proteose peptone acid medium, proved very satisfactory both for the detection of the organism in spoiled tomato juice and for its subsequent cultivation in the laboratory. The final pH of the medium after sterilization was about 5. This acidity, although inhibitive for most of the nonsignificant types of organisms found in tomato juice, was in the optimum pH range for *B. thermoacidurans*.

Temperature of Incubation: Numerous comparisons have shown that for routine examination of *B. thermoacidurans* in tomato juice, there is a slight advantage to incubation of the organism at 55°C. on proteose peptone acid medium. Growth in tomato juice of an acclimatized culture occurs both at 37°C. and at room temperature, but not at 55°C. For heat resistance studies it has been found that higher counts of recovery cultures were obtained when the plates were incubated at 37°C. rather than at 55°C. We have, therefore, adopted the routine of incubating all cultures on artificial medium at 55°C., except recovery cultures from heat resistance runs which are incubated at 37°C., and of incubating all inoculated juice at either 37°C. or room temperature.

Laboratory Care and Cultivation of B. thermoacidurans: Proteose peptone acid agar proved to be a satisfactory medium for the laboratory cultivation of *B. thermoacidurans*. Abundant crops of vegetative cells as well as spores can be obtained by use of this medium.

It has been observed, both in this laboratory and elsewhere, that after several transfers in nutrient broth, cultures of *B. thermoacidurans* would not cause spoilage of normal tomato juice. Only organisms cultivated in tomato juice apparently retained the ability to spoil normal juice.

A series of experiments was carried out to determine the influence of various methods of cultivation on the ability of *B. thermoacidurans* to grow in and to spoil tomato juice.

Transfers of the organism were made from spoiled tomato juice into proteose peptone acid agar and proteose peptone acid broth, into nutrient agar and nutrient broth, and into normal tomato juice. All cultures were incubated at 37°C. After 24 hours growth was readily visible in the proteose peptone acid broth and on the proteose peptone acid slants, but only scant growth was observed on the nutrient agar and in the nutrient broth. After five days' incubation abundant growth was present in the tubes of proteose peptone acid agar and broth, but little growth was evident in the nutrient broth and on the nutrient agar. Microscopic observation of growth from proteose peptone acid agar and broth revealed the presence of long slender rods with a definite tendency to chain. No spores were present on any of the smears.

After two weeks' incubation considerable sporulation had taken place on the proteose peptone acid agar slants. Some few spores also were observed on the nutrient agar, but in the nutrient broth, in the proteose peptone acid broth, and in the tomato juice tubes only vegetative cells were present.

At this time suspensions were made in sterile, distilled water from growth on proteose peptone acid slants and nutrient agar slants, and half of each suspension was boiled for five minutes to kill all vegetative cells. The broth cultures were boiled similarly. Inoculations into normal tomato juice then were made from these heated and unheated suspensions and also from spoiled tomato juice. All of the inoculated tubes were incubated at 37°C. for three weeks at which time pH determinations, organoleptic tests, and microscopic examinations were made.

That only those organisms grown previously on proteose peptone acid agar and in tomato juice, and to a lesser degree those organisms grown in proteose peptone acid broth, were able to spoil normal tomato juice is clearly shown (Table 1). Greatest activity apparently took place in the juice inoculated from previously spoiled juice and in the juice inoculated from a heated suspension prepared from a proteose peptone acid slant. The pH of the juice from these tubes was 3.91 and 3.93, respectively, as compared with a pH value of 4.2 for normal juice. The drop in pH was not as great in the juice inoculated from proteose peptone acid broth, but considerable numbers of cells were present, and the spoiled juice had the typical off-flavor associated with this type of spoilage. None of the juice inoculated from cultures grown in nutrient broth or on nutrient agar showed any activity. It is also interesting to note that no growth was apparent in the juice inoculated from a heated suspension prepared from a proteose peptone acid broth culture. It previously was observed microscopically that no spores were present in the broth culture. Therefore, since all vegetative cells were killed by boiling, it is reasonable to expect that no growth would take place in tomato juice inoculated from this culture.

To date cultures have been carried through five transfers on the different types of media. At the time of each transfer tubes of normal tomato juice were inoculated as described above. In all cases the results obtained agree substantially with those given (Table 1). Occasionally normal juice inoculated from a nutrient agar slant will spoil, but this occurrence is rare and is not predictable.

TABLE 1

Influence of Different Methods of Cultivation on Growth and Activity of Bacillus thermoacidurans in Tomato Juice

Type of inoculum used	pH after 3 weeks	Microscopic examination of tomato juice	Taste
Heated suspension from nutrient agar	4.18	No cells present	Normal
Total growth from nutrient agar	4.18	No cells present	Normal
Heated suspension from nutrient broth	4.20	No cells present	Normal
Total growth from nutrient broth	4.16	No cells present	Normal
Heated suspension from proteose peptone acid agar	3.93	Long chains of rods, but no spores present	Sour
Total growth from proteose peptone acid agar	3.97	Long chains of rods, but no spores present	Sour
Heated suspension from proteose peptone acid broth	4.17	No cells present	Normal
Total growth from proteose peptone acid broth	4.10	Some rods, but no spores present	Sour
Spoiled tomato juice	3.91	Long chains of rods, but no spores present	Sour

In another experiment organisms were transferred to proteose peptone acid agar slants and incubated for two weeks at 37°C. At this time the cultures, all of which showed considerable sporulation, were sealed in an oxygen flame and stored in the refrigerator. After a month's storage one of the tubes was broken and several transfers were made into normal tomato juice. At the end of three weeks the typical off-flavor was present in the juice, and the pH had dropped from 4.22 to 3.97. Similar tests with cultures stored in the refrigerator were made the three following months, and in all three instances the organisms retained their ability to grow in and to spoil normal tomato juice. It is planned to continue this series of experiments for a longer period of time.

Detection of B. thermoacidurans in Tomato Juice: Preliminary investigations indicated that plating methods could be applied most satisfactorily for the detection of *B. thermoacidurans* in tomato juice. The organisms can be plated successfully directly from the juice since there are rarely any contaminating types and since the colonies produced by the organisms are identified easily. During the course of study of this problem it has been observed consistently that unless *B. thermoacidurans* was present in the sample of tomato juice the plates appeared sterile. The application of a plating technique also is advantageous because the results obtained give both a qualitative and quantitative picture of a spoilage case.

Experimentation revealed that tomato juice has an inhibiting effect on the growth of the organisms in the plating medium. The addition of one

ml. of normal tomato juice reduced the count by 22.5 per cent, and the addition of three ml. of juice reduced the count by 31.3 per cent (Table 2). It was decided on this basis to plate out only one ml. of the juice for a routine test. This would reduce the inhibiting action to a minimum and would allow the results to be reported, without further calculations, as numbers of bacteria per milliliter.

TABLE 2

Influence of Added Tomato Juice on Growth of Bacillus thermoacidurans on Proteose Peptone Acid Agar

Petri dish No.	No juice added	1 ml. of juice added	3 ml. of juice added
	bacteria per ml.	*bacteria per ml.*	*bacteria per ml.*
1	460	341	306
2	437	366	292
3	445	335	323
4	463	358	322
Av. count per ml.	451	350	310
Count as pct. of control plates	100	77.5	68.7

The technique finally adopted for the detection of *B. thermoacidurans* in tomato juice consists of opening the can aseptically, placing one ml. of the juice in each of three sterile Petri dishes, and pouring into each dish approximately 20 ml. of proteose peptone acid agar at 45 to 55°C. After the plates have hardened, they are incubated at 55°C. for 48 hours and then counted. Supplementary to the counts pH determinations as well as organoleptic examinations are made on the spoiled juice.

SUMMARY

A culture of *B. thermoacidurans* was acclimatized by repeated transplanting from tomato juice to tomato juice until spoilage would always develop upon inoculation into normal juice.

Of a large number of ingredients tested, a combination containing proteose peptone, yeast extract, glucose, and K_2HPO_4 at pH 5 proved to be a satisfactory medium for the cultivation of *B. thermoacidurans*. Abundant crops of vegetative cells and of spores were obtained by the use of this medium.

Continued transfer of *B. thermoacidurans* on nutrient agar produced cultures that were unable to grow in and spoil normal tomato juice. On the other hand, continued transfer of the organisms on proteose peptone acid agar had no apparent influence on the ability of the organism to spoil tomato juice. After four months' storage in the ice box, sealed cultures on proteose peptone acid agar of *B. thermoacidurans* were still viable and were still able to cause spoilage in tomato juice.

For the detection of *B. thermoacidurans* in tomato juice a plating technique employing proteose peptone acid agar was found to be most satisfactory because the results obtained give both a qualitative and quantitative

picture of a spoilage case. Since tomato juice was observed to have an inhibiting effect on the growth of *B. thermoacidurans,* only one ml. of juice was plated out in 20 ml. of medium.

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THE COMMON HOUSE FLY *(MUSCA DOMESTICA)* AS A SOURCE OF POLLUTION IN FOOD ESTABLISHMENTS

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The widespread occurrence of the common house fly *(Musca domestica)* has led numerous investigators to study these insects as potential vectors of bacteria. Although flies are thought to play a certain, though not extensive, role in the transmission of organisms identified with filth and disease, the significance of numbers of flies in and around food establishments has not been correlated with their ability to pollute or contaminate food in preparation or storage.

Marpmann (1884) pressed a drop from the proboscis and anus of 230 flies, examined this material in stained preparations, and established the presence of microörganisms in every fly. Horrocks (1903), Graham-Smith (1909), Nicoll (1911), and Cox, Lewis, and Glynn (1912) isolated many strains of colon-aerogenes bacteria from the surface and intestinal tract of flies and came to the general conclusion that colon-aerogenes bacteria, Morgan's bacillus No. 1, and many strains of slow lactose fermenters are common inhabitants of the intestinal tract of these insects. Further investigations by Ficker (1902), Hamilton (1903), Faichnie (1909a, b), Graham-Smith (1909), Ledingham (1911), Nicoll (1917), Bahr and Comb (1914), and Manson-Bahr (1919) demonstrated the presence of *Eberthella typhosa, Salmonella enteritidis, Vibrio cholerae, Salmonella schottmülleri*, and several *Shigella* strains in the intestinal tract. These pathogens were reported to have persisted in the digestive system for from two to 23 days after infection. In practically every instance these microörganisms were found in flies caught in epidemic areas. It was generally concluded that adult flies were a health menace and that they were capable of spreading countless numbers of pathogens by defecation or regurgitation. In a recent study Sanders (1940) transmitted bovine mastitis from diseased to normal cattle by allowing flies which had fed on infected quarters to come in contact with the quarters of healthy cows. Bacot (1911), Tebbutt (1913), and Glaser (1923) studied the question of bacterial survival during metamorphosis of *Musca domestica* and found that bacteria could survive from larvae to adult fly. These investigators felt, however, that there was only a remote possibility of infection with pathogenic organisms in flies emerging from pupae which had developed in infected breeding grounds.

EXPERIMENTAL PROPAGATION OF FLIES

In order to secure some definite facts as to the tendencies and possibilities of flies to act as vectors of microörganisms, a series of experiments were conducted on flies developed in the laboratory, following in general the method of propagation outlined by Campbell and Sullivan (1938). The method consists of obtaining eggs on cotton soaked with equal parts of milk

and water in a Petri dish placed in the fly cage. Within approximately 24 hours clusters of eggs, deposited in the folds of cotton, are removed with the aid of a penknife or similar instrument and suspended in physiological salt solution in order to break up the small clumps. The eggs are allowed to settle and a volume of from 1.0 to 1.5 c.c. is resuspended and poured on the surface of Richardson's medium (1932) in a large battery jar.

The medium in each jar is composed of 1.2 kg. of bran-alfalfa leaf meal (2:1 by weight) into which is poured and kneaded a mixture consisting of 40 c.c. of a commercial malt syrup, 150 c.c. of a yeast suspension, and 2.5 liters of water. It is necessary to guard against an excessive amount of water or too few developing maggots in the medium as either will result in

FIG. 1. Trap for catching adult flies emerging from pupae in the top, dried-out layers of mash.

an overgrowth of mold which may retard or completely destroy maggot development.

Maggots may be allowed to pupate in the top dried-out layers of this synthetic medium. Pupation is readily accomplished if a cover consisting of cheesecloth-covered absorbent cotton is placed over the battery jar. An expedient method for trapping flies as they emerge from the pupae is to cut a small hole in the center of the cheesecloth cover and to place over it the open rubber back of the cage (Fig. 1). A more desirable method of obtaining adult flies is to allow the maggots to crawl over the edge of the battery jar and into a pan of sifted sawdust where pupation occurs (Fig. 2). If this method is used, it is necessary to place a glass cover over the top of the battery jar to preserve moisture, the cover being small enough to leave a slight opening on both sides of the top of the battery jar. Pres-

ervation of moisture may also be accomplished by elevating the cover by means of paper clips. In 24 to 48 hours the maggots will crawl over the wall of the battery jar and drop into the sawdust to pupate. This method of development permits the removal of known numbers of uniform-sized pupae. Further development of these pupae may be retarded for short periods of time by holding them in cold storage. The population of adults formed from selected pupae are uniform in size and may be assumed to have uniform vigor and resistance. The initial flies used in these experiments were from a population two generations removed from any contact with fecal matter, and during these studies approximately 12 plantings of eggs were made, the adults of which were involved in these experiments.

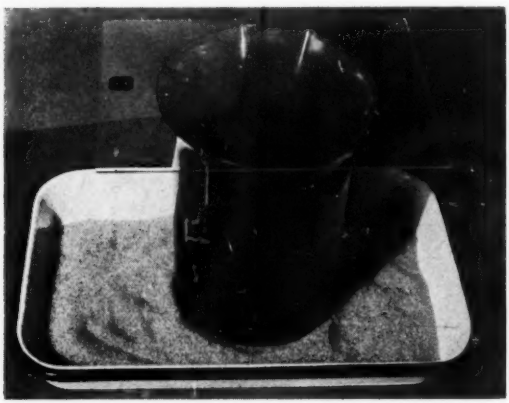

FIG. 2. Method of developing pupae. Maggots crawl over edge of jar and fall into sawdust where pupation occurs.

Food for the adult flies consisted of equal parts of sterile milk and water and, occasionally, sucrose solution consisting of a lump of table sugar dissolved in 10 c.c. of water.

METHODS AND RESULTS OF EXAMINING FLIES FOR SURFACE ORGANISMS

Caged flies are caught for bacteriological examination by inserting a wide-mouth, rubber-stoppered flask into the cage through the opening in the rubber back (Fig. 3). The tight closure of the rubber around the arm of the trapper prevents any escape of flies. The flask is held directly over a resting fly and as it is disturbed it usually flies directly up into the flask. The rubber stopper is placed inverted on the floor of the cage, and after the desired number of flies are trapped the stopper may be replaced and the inverted flask removed.

All flies were anesthetized with ether, washed with known amounts of sterile physiological salt solution, and the washings planted in decimal dilutions in triplicate in nutrient and tryptose agar for total bacterial counts. The Petri dishes were incubated at 37°C. (98.6°F.) for 48 hours. From each dilution transplants were also made into standard lactose broth and incubated at 37°C. for 24 hours. At the end of the 24-hour incubation period

all positive presumptive lactose broth tubes were streaked on Levin's eosin methylene blue agar. These plates were likewise incubated at 37°C. for 24 hours, at the end of which time representative colonies were fished for purification and identification according to procedures given in Standard Methods of Water Analysis (1936).

Washings from the surface of the test flies yielded total bacterial counts from 2,500,000 to 29,500,000 per fly. Although the greatest number of organisms was found on the surface of the flies in the first few generations developed in the laboratory, succeeding generations did not show marked

FIG. 3. Method of collecting flies for study.

reductions in total counts. It should be noted, however, that these flies were not propagated in sterile media and were originally developed from maggots obtained from a manure pile.

Colon-aerogenes bacteria were present in dilutions representing at least 1/1,000,000 c.c. of the washings in the first few generations developed. Typical and atypical forms of *Escherichia coli*, *Aerobacter aerogenes*, and intermediates were present on the surface of the flies throughout the entire period of these studies, and although no attempt was made to establish the numerical frequency of the occurrence of the various colon-aerogenes types, it was noted that the number of typical *Escherichia coli* types diminished as the generations of adult flies became further removed from any contact with fecal matter.

METHODS AND RESULTS OF EXAMINING FLIES FOR INTESTINAL ORGANISMS

The washed flies used for determining surface bacteria were utilized for the determination of intestinal organisms. In order to avoid surface con-

taminants, attempts were made to sterilize the surface of the flies by the use of various chemical disinfectants. Although previous investigators, Nicoll (1917) and Ledingham (1911), reported that two per cent lysol in absolute alcohol for a period of 10 to 20 minutes, or 1:1000 potassium mercuric chloride, were used successfully for destruction of surface bacteria on flies, we were unable to confirm these findings. Furthermore, the application of 1:1000 potassium mercuric iodide for 10 minutes; 80 per cent alcohol for ten minutes; five per cent soap solution for 15 minutes followed by 80 per cent alcohol for 15 minutes; and a mixture of one per cent sodium hydroxide, one per cent aerosol, and five per cent formaldehyde for 10 minutes, all were ineffective in bringing about complete sterilization. Invariably, following treatment with the above chemicals, spore-forming organisms could still be demonstrated on the surface of the flies under examination. In every instance, the first washing following chemical treatment failed to show any evidence of viable bacteria, but it was obvious that this was the result of bacteriostatic action of the chemical rather than an absence of microörganisms, since in subsequent washings viable bacteria could be demonstrated. However, in view of the fact that washing for 10 minutes in a mixture of one per cent sodium hydroxide, one per cent aerosol, and five per cent formaldehyde not only destroyed vegetative forms but materially reduced the number of spore formers, this solution was used in subsequent experiments. As a check on the germicidal efficiency of the chemicals used, following treatment, the flies were washed from three to five times with nutrient broth as well as lactose broth and the washings incubated at 37°C. for 24 and 48 hours. All washed flies were then ground in a sterile mortar and total bacterial counts were made of the residue on the basis of organisms per fly and the number of colon-aerogenes bacteria determined.

After chemical treatment the macerated flies showed total bacterial counts of from 84,000 to 2,000,000 per individual, and colon-aerogenes bacteria were demonstrated in dilutions representing 1/100 c.c. The frequency of the occurrence of various members of the colon-aerogenes bacteria obtained from the internal organs followed the trend of those found on surface washings except that all types were present in much fewer numbers. As was found with examination of surface washings, typical *Escherichia coli* types diminished as the succeeding generations of adult flies became further removed from contact with fecal matter.

FOOD POLLUTION

To establish actual pollution of food by fly contact, six 50-gram portions of sterilized pecan meats in sterile Petri dishes were exposed to approximately 150 flies for varying periods of time. These flies were obtained from a larger population which for three generations had been caged with six mice infected with *Salmonella enteritidis*. The pecans were removed and examined at the end of one-fourth, three-fourths, one and one-half, four, and five hours. It will be noted (Table 1) that at the end of the 15-minute interval 900 bacteria had been deposited per gram of pecans. In 45 minutes the bacterial count increased to 560,000 per gram, and, except for the one and one-half hour interval when the total count was 200,000 per gram, there

was a gradual increase in the number of bacteria deposited until the fifth hour when counts of 3,500,000 per gram of pecans were obtained. *Salmonella enteritidis* was isolated from the pecans in all the intervals of exposure. Further work with this organism will be reported at a later date.

A minimum of 10 *Escherichia coli* per gram of pecans was deposited in 15 minutes. The number of these bacteria deposited progressively increased with increased periods of exposure, 100,000 *Escherichia coli* having been deposited on the pecans exposed for four hours. Although no *Escherichia coli* were isolated from any of the washings of pecans exposed for five hours, colon-aerogenes bacteria giving IMVIC reactions for intermediates were

TABLE 1

Contamination of Pecans by Flies

Time exposed to flies	1 gram	.1 gram	.01 gram	.001 gram	.0001 gram	.00001 gram	Total count per gram	*S. enteritidis*
hr.								
¼	Pseudo.[1]	E. coli	.....	.....	.....	.....	.....	Positive[2]
	Inter.	.....	.....	.....	.....	.....	.....	.....
	E. coli	.....	.....	.....	.....	.....	900	.....
¾	Pseudo.	Pseudo.	Inter.	Inter.	.....	.....	.....	.....
	E. coli	E. coli	E. coli	E. coli	.....	.....	560,000	Positive
1½	Pseudo.	Pseudo.	Pseudo.	Pseudo.	Inter.	Inter.	200,000	Positive
				Inter.	Aerog.			
					E. coli			
2½	.....	Pseudo.	Pseudo.	Pseudo.	Pseudo.	Pseudo.	760,000	Positive
4	.....	Pseudo.	Pseudo.	Pseudo.	Pseudo.	Inter.	1,500,000	Positive
			Inter.			Aerog.		
			E. coli			E. coli		
5	.....	Pseudo.	Pseudo.	Pseudo.	Pseudo.	Pseudo.	3,500,000	Positive
						Inter.		

[1] Pseudo. = *Pseudomonas aeruginosa*; Inter. = Intermediates (colon-aerogenes); E. coli = *Escherichia coli*; Aerog. = *Aerobacter aerogenes*. [2] *S. enteritidis* was identified biochemically and serologically.

recovered from the dilutions representing 1/100,000 gram of pecans. Decimal dilutions in lactose broth of 1/10 to 1/10,000 of the washings from the pecans exposed for five hours contained pure cultures of *Pseudomonas* and it appeared that our inability to isolate *Escherichia coli* in these dilutions was due to overgrowth by this organism.

It is, perhaps, noteworthy to mention that throughout all these studies, and particularly so in the pecan pollution experiments, *Pseudomonas aeruginosa* was invariably encountered and of the 54 plates streaked in the above experiment, more than one-third contained pure cultures of this organism.

DISCUSSION

The problems relating to sanitation in food-producing establishments have long been a subject of investigation. The effect of providing and maintaining adequate facilities for personal hygiene among food operators, the need for supervision of operations and for adequate cleansing of equipment, and the effect of rodent infestation have all been clearly defined. Chow

(1940) has clearly demonstrated that flies are a menace to public health. He trapped 650 adult flies *(Chrysomyia megacephala)* in Peiping, China, from public latrines, garbage containers, and fruit and vegetable stands and found that from 80 per cent to 100 per cent of these insects contained *Escherichia coli*. Eight per cent of the flies examined contained *Shigella dysenteriae*. *Eberthella typhosus* and *Shigella dysenteriae* survived on or inside the body of artificially infected flies for from five to six days. The results of our investigations (to be published) with flies *(Musca domestica)* artificially infected with *Salmonella enteritidis* indicated that these pathogens survive for the duration of life of the fly.

Because of the external hairy anatomical structure of house flies and the fact that they indiscriminately breed in and inhabit every conceivable source of filth, these insects are capable of being mechanical carriers of countless numbers and types of bacteria, the external and internal bacterial flora depending largely on the specific places and materials the flies inhabit. In a study of dispersion of flies by flight, Bishopp and Laake (1921) found that *Musca domestica* had a maximum distance spread of 13.14 miles and that the main stimulus inducing dispersion was the desire for food. Temporary exposure of refuse in urban areas and the frequency of outhouses, dumps, and domestic animal excrement in rural areas constitute sources of filth conceivably within range of flight of flies regardless of the location of the breeding grounds. With such a far-reaching range of flight, the presence of a maximum of 29,500,000 bacteria on the surface of flies, bred and fed under controlled conditions, might conceivably represent the minimum numbers present on flies in nature. The presence of at least one million colon-aerogenes bacteria on the surface of a fly, housed under laboratory conditions, might likewise represent the minimum numbers present on flies having access to polluted food. There would also be less likelihood of finding diminishing numbers of *Escherichia coli* in flies found in nature than in flies fed and bred under laboratory conditions.

The ability of flies to contaminate food is clearly demonstrated from the results obtained when sterile pecans are exposed to flies, since within a very short time large numbers of organisms are deposited. It is of particular significance that throughout the six periods of exposure (15 minutes to five hours) not more than 12 flies were seen to be feeding on the pecan meats at any one time, and on the average, only eight flies were present during 11 counts made at random of the number of flies on each of the dishes of exposed pecans. These flies, which were carriers of *Salmonella enteritidis*, deposited this organism on the pecan meats in every period of exposure.

Whereas an average of 100,000 colon-aerogenes bacteria was deposited on the pecan meats in five hours, it should be noted that there was little, if any, possibility of multiplication of these organisms. In a previous publication by Ostrolenk and Hunter (1939) it was shown that contamination of pecan meats with colon-aerogenes bacteria remains constant for short periods of time and decreases over long periods of storage. By contrast it seems safe to assume that 100,000 colon-aerogenes bacteria deposited on a moist product, such as crab meat, not immediately refrigerated, would in a five-hour period yield considerably larger numbers of these bacteria.

The results of these studies indicate that flies are the potential sources of filth, that they have previously been suspected, and that the presence of even small numbers of flies in a food establishment constitutes a filth menace the seriousness of which may be determined by careful evaluation of surrounding conditions.

SUMMARY AND CONCLUSIONS

The methods employed in rearing and examining flies in the laboratory are given.

Washings from the surface of flies contained 2,500,000 to 29,500,000 bacteria per fly. Colon-aerogenes bacteria are present in amounts representing 84,000 to 2,000,000 bacteria, *Escherichia coli* being present in considerably reduced numbers.

The incidence of colon-aerogenes bacteria externally and internally depends largely on the material on which flies feed.

Flies may act as carriers of both pathogenic and saprophytic bacteria and are capable of depositing large numbers of these organisms on food in very short intervals of time.

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KINETICS AS A FUNCTION OF TEMPERATURE OF LIPASE, TRYPSIN, AND INVERTASE ACTIVITY FROM —70 TO 50°C.(—94 TO 122°F.)

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The functioning of enzymes at temperatures below 0°C.(32°F.) has long been recognized by those interested in cold storage of foods where it appears that the chief changes which occur are enzymatic rather than due to bacterial growth—Haines (1937), Banks (1937), Clark and Philipson (1931), Diehl, Dingle, and Berry (1933), Prescott, Bates, and Highlands (1932), and Reed (1935). These observations were purely of a qualitative nature, and there has been little quantitative work on enzyme kinetics at low temperatures, although such a study might be expected to contribute much to our knowledge of enzymes as well as to furnish information of fundamental importance in the cold storage of foods. In this work three typical enzymes —pancreatic lipase, trypsin, and yeast invertase—which attack fats, proteins, and carbohydrates, respectively, have been selected for study. The functioning of lipase at low temperatures has been recognized as responsible for undesirable flavors developed in cream and fats at low temperatures by Anderson (1937), Bosco (1937), and Lea (1930). Balls, Lineweaver, and coworkers (1937-1939) have shown that lipases hydrolize olive oil and tributyrin with considerable velocity even when the system is frozen. Proteases also function at low temperatures as is indicated by hydrolytic changes which occur during the cold storage of meats and fish, Tressler (1933); eggs, Balls and Swenson (1934); and milk and cream, Anderson (1937). Balls and Tucker (1938) studied tissue proteases and found that protein hydrolysis occurs with increasing slowness as the temperature is lowered, and after the system freezes the action becomes extremely retarded. Little work on the function of invertase at low temperatures has been reported, although changes in the syrup of figs occurs after storage at —18°C. (—.4°F.), Reed (1935); and sucrose inversion occurs in frozen plums, Tressler and Evers (1936); and in certain frozen fruits and vegetables, Tressler (1933). Kertesz (1933) reported that invertase is active at temperatures below 0°C. and has made a comparison of the kinetics of invertase action both above and below 0°C.

From both theoretical and practical viewpoints it seems important to make a study of typical enzymes not only in the temperature range below 0°C. but also in the range above this temperature, in order to determine whether there is any abrupt change in kinetics at 0°C. or at the freezing point of the digest. This problem has considerable importance in the food industry since, if there is no such change in kinetics over this range, it is

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possible to calculate enzyme action at low temperatures from a knowledge of the kinetics at higher temperatures, Lineweaver (1939).

TEMPERATURE CONTROL

For temperatures between 50 to 0°C.(122 to 32°F.) use was made of a water bath with temperature controlled to ± .05°C. For temperatures from 0 to —25°C.(32 to —13°F.) a specially insulated gas refrigerator with temperature controlled to ± 1.0° was satisfactory. The temperature of —70°C.(—94°F.) was attained with solid CO_2 kept in a well-insulated refrigerator. All temperatures below 0°C. were measured with a calibrated thermocouple.

LIPASE

The method of Balls, Lineweaver, *et al.* (1937-1939) for determining lipase activity was used in this study. Two series of experiments were made, one with 36.5 per cent glycerol in the digest (freezing point —18.5°C.) and

TABLE 1

Hyrdolysis of Tributyrin by Pancreatic Lipase in Presence and Absence of Glycerol in the Digest

°C.	Glycerol present	Glycerol absent
	Time for 1% hydrolysis	Time for 1% hydrolysis
	hr.	*hr.*
50.0	0.0183	0.020
40.0	0.0198	0.025
30.0	0.0325	0.040
20.0	0.0850	0.050
10.0	0.0800	0.100
5.0	0.1330	0.133
0.2	0.1370	0.150
—4.5	0.5500	1.000
—4.5	0.4000	1.400
—5.0	0.5000	1.050
—9.0	1.6200	2.750
—10.0	8.5000	4.000
—12.0	4.0000	5.000
—15.0	3.5000	10.500
—18.0	7.0000	20.000
—18.0	4.0000	9.500
—18.0	7.5000	7.500
—24.5	87.5000	121.000
—24.5	55.0000	200.000

one with water instead of glycerol in the digest (freezing point —3°C.). The data of these two series permitted a comparison to be made in the temperature range from —3 to —18.5°C.(26.6 to —1.3°F.) between the kinetics of the lipase system in the liquid (glycerol present) and in the solid state (no glycerol present).

The lipase solution was prepared by dissolving one gram of pancreatin (Armour) plus 3.5 gm. bile salts (Fairchild) plus .75 gm. $CaCl_2$ in 75 ml. .05 M NH_4Cl. To this were added 50 ml. glycerol or water, seven drops of three per cent phenolphthalein, two ml. water, and finally six N

NH_4OH by drops until the pH was 8.1. At the beginning of the experiment 10 ml. tributyrin (Eastman) were added to this solution. Both enzyme solution and substrate were adapted to temperature before being combined at temperatures above 0°C. Five-ml. portions of the mixture were then placed in test tubes. For studies below 0°C. the solutions were cooled to 0°C. and then combined. Then five-ml. portions were placed in a series of small tubes which were adjusted rapidly to the desired temperature by surrounding them with powdered solid CO_2. At successive time intervals duplicate five-ml. samples were treated with 70 ml. of a mixture of nine to one alcohol and ether to stop the reaction. The free fatty acid liberated during the hydrolysis was then titrated with .1 N alcoholic KOH, prepared according to McCallum (1921). For work below the freezing point of the

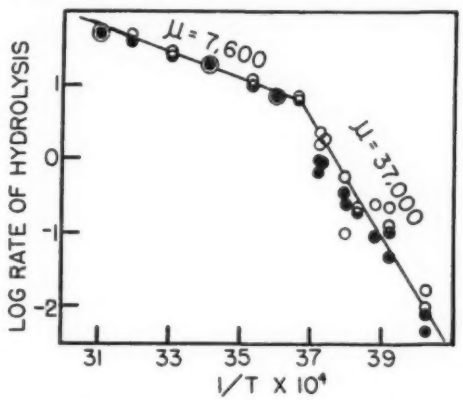

FIG. 1. Log rate of hydrolysis (i.e., log reciprocal of time required for one per cent hydrolysis) of tributyrin by lipase is plotted against the reciprocal of the absolute temperature.

⊙—glycerol present in the digest.

●—no glycerol present in the digest.

digest the alcohol-ether mixture was added to the test tube and the digest thawed out by placing the tube in hot water. The digest was thus melted rapidly in a medium which stopped the enzyme action.

At all temperatures from 50 to —70°C. the hydrolysis of tributyrin by lipase was studied as a function of the elapsed time and it was found that the reaction follows a smooth curvilinear course—Tauber (1937), Sobotka and Glick (1934), and Weinstein and Wynne (1936). The reciprocal of the time required to bring about one per cent hydrolysis of the fat was taken as a measure of the rate at each temperature (Table 1). When log rate is plotted against the reciprocal of the absolute temperature (Fig. 1), the points fall roughly along two straight lines which intersect at 0°C. This indicates that the rate of fat hydrolysis increases exponentially with temperature in accordance with the Arrhenius equation

$$\mu = 4.56 \frac{(\log R_2 - \log R_1)}{1/T_1 - 1/T_2}$$

where R_1 and R_2 are the rates at the absolute temperatures T_1 and T_2 and μ is the energy of activation of the reaction in calories per gram mol. The slope of the straight line drawn through the points for the higher temperature range corresponds to a μ value of 7,600 cal., while for the other curve the corresponding value is 37,000 cal.

The sharp break in the temperature relationship at 0°C. is very striking and indicates that the kinetics of lipase action are very different above and below this point. Although the data are much more scattered in the lower temperature range, it is evident that they are none the less in accord with the Arrhenius equation. This break occurs at about the same temperature for both the digest containing glycerol and that without glycerol. This is surprising since the change of state from liquid to solid occurs at about this point only with the system without glycerol; in the system with glycerol freezing occurs at —18.5°C., yet the break in the curve appears at 0°C., not at —18.5°C.

It is apparent from these data that lipase has appreciable activity even at temperatures as low as —25°C.(—13°F.). No significant activity was noticed at —70°C., however, in 27 days. Independent tests showed that storage of lipase at —70°C. did not inactivate the enzyme. At about 50°C., however, inactivation of the enzyme by heat becomes apparent (Fig. 1).

The μ value of 7,600 cal. for lipase at normal temperatures agrees well with values calculated from data of Terroine (1910) and Nicloux (1904) but is not in accord with that calculated from data of Kastle and Loevenhart (1900). The data reported in this paper on lipase activity in the presence of glycerol at low temperatures agree qualitatively with Balls and Lineweaver (1938) who report no sharp change in rate corresponding to change in state from liquid to solid at —12°C.(10.4°F.).

TRYPSIN

The course of tryptic digestion of casein was followed by formol titrations, Cole (1933), of the free carboxyl groups liberated during the hydrolysis. A 10 per cent solution of Hammarsten's casein (Merck) was made up in a solution containing .05 per cent NaOH so that the final pH was approximately 8.1. A five-per cent solution of hog trypsin (Central Scientific Co.) was used as the enzyme solution. Twenty ml. of the trypsin solution were added to 100 ml. of the casein solution to initiate hydrolysis. The adaptation to high and low temperatures was performed as in the lipase experiments. Five-ml. samples were taken for analysis at successive time intervals and five ml. of 40 per cent formaldehyde were added to stop the reaction. Then the solution was titrated to pH 8.1 with .1 NaOH.

Typical smooth curves, Tauber (1937), are obtained when casein hydrolysis, as measured by formol titration, is plotted against the duration of the digestion. The rate of the reaction was measured by the reciprocal of the time required to liberate .0001 or .0003 mol of acidic carboxyl groups (Table 2). An Arrhenius plot of the data (Fig. 2) indicates that the reaction follows the Arrhenius equation with a sharp break at about the freezing point of the digest, —1.5°C.(29.3°F.), and corresponding energies of activation of 15,400 cal. for the upper and 65,000 cal. for the lower temperature range. As in the case of lipase the 50°C. point is low because

of partial heat inactivation. No measurable hydrolysis in one month occurred at —70°C., although there is no inactivation of trypsin at this temperature. Balls and Lineweaver (1938) observed no trypsin inactivation at liquid air temperature.

TABLE 2

Hydrolysis of Casein by Trypsin as Measured by Formol Titration of Acidic Carboxyl Groups Liberated During Reaction

°C.	Time for liberation of .0001 mol of acid	Time for liberation of .0003 mol of acid
	hr.	*hr.*
50.0	0.05	0.18
40.0	0.06	0.24
30.0	0.13	0.44
20.0	0.25	1.33
10.0	0.80	2.82
0.2	2.10	7.43
—4.5	7.50	.....
—10.0	210.00	.....
—15.0	750.00	.....

As with lipase, the data follow the Arrhenius equation at all temperatures, although the kinetics of both enzymes are very different in the range of low temperatures from the situation at high temperatures. The activation energies reported for tryptic hydrolysis above 0°C. are inconsistent—

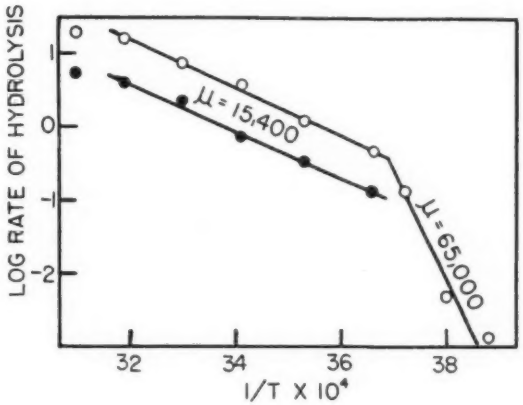

FIG. 2. Log rate of hydrolysis of casein by trypsin is plotted against 1/T.
⊙—rate calculated from the time required to liberate .0001 mol of acid.
●—rate calculated from the time required to liberate .0003 mol of acid.

Lineweaver (1939), Barnes (1937), Buchanan and Fulmer (1930), Arrhenius (1915), Vernon (1904), Moelwyn-Hughes, Pace, and Lewis (1930)—and in general not closely comparable to the value reported here. Balls and Lineweaver (1938) studied tryptic action at high and low temperatures, down to —1.0 and —6.7°C. (30.2 and 20°F.), with and without

added glycerol and obtained μ values of 12,000 cal. and 20,000 cal. (calculated from their data) with no break occurring at 0°C. Their data do not extend to sufficiently low temperatures, however, to be certain about such a break.

INVERTASE

The hydrolysis of sucrose by yeast invertase was followed by measuring the amount of invert sugar liberated, utilizing for this purpose a colorimetric method for reducing sugars employing Sumner's dinitrosalicylic acid reagent, Sumner (1925) and Sumner and Howell (1935), as performed by Sizer (1938a, b). A stock sucrose solution was freshly prepared which contained six per cent sucrose and was buffered to pH 4.45 with M/5 KH_2PO_4. A one-per cent solution of liquid yeast invertase (Digestive Ferments Co.) was the enzyme preparation used. Twenty ml. of the sucrose solution were mixed with four ml. invertase to initiate the reaction after temperature

TABLE 3

Hydrolysis of Sucrose by Invertase as Measured by Amount of Invert Sugar Liberated During Reaction

°C.	Time for liberation of 1 mg. invert sugar, hr.
50.0	0.0333
40.0	0.0312
30.0	0.0583
20.0	0.1170
10.0	0.2250
0.2	0.3750
—4.5	1.8700
—10.0	5.0000
—15.0	80.0000
—18.0	520.0000

adjustment had been made. At all temperatures the technique was the same as for the lipase experiments except that one-ml. amounts were placed in tubes. At successive intervals duplicate one-ml. samples were treated with three ml. dinitrosalicylic acid reagent which stopped the inversion instantly. The solutions were treated according to Sumner and then compared in the colorimeter with standard glucose solutions prepared in a similar manner.

Smooth curves were obtained when milligrams of invert sugar liberated during the hydrolysis were plotted against the duration of the experiment. The curves appear identical with those of Sizer (1938a, b) who reported that over the first third of the reaction inversion follows the monomolecular equation. The reciprocal of the time required to liberate one mg. of invert sugar was taken as a measure of rate at each temperature (Table 3). When log rate is plotted against 1/T the data fit along two straight lines which intersect at about —2°C. (28.4°F.). The corresponding activation energies are 11,100 cal. per gram mol above —2°C. and 60,000 cal. below this temperature. The point for 50°C. is low because of partial heat inactivation of the enzyme. As in the case of lipase and trypsin, the data fit the Arrhenius equation at all temperatures below 40°C., but a sharp transition occurs at —2°C. with a much higher activation energy characterizing the

system over the lower temperature range than the higher. No measurable inversion occurred at —70°C. in 27 days, although no inactivation or change in activity of the invertase occurred at that temperature.

The activation energy of 11,100 cal. characterizing the system above 0°C. agrees with those reported by Sizer (1937, 1938a, b), Buchanan and Fulmer (1930), and Arrhenius (1915) but disagrees with some of the values in the older literature—cf. Sizer (1937, 1938a). Kertesz (1933), employing supercooled solutions, worked with invertase at temperatures down to —7.5°C. (18.5°F.) but his data are not in accord with the Arrhenius equation, and in fact indicate that low temperatures are not very effective in retarding invertase activity. Lineweaver (1939) has made use of Sizer's data for inversion above 0°C. and calculated inversion rates for lower temperatures.

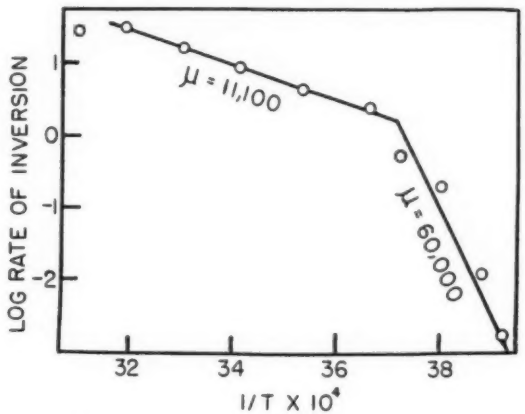

FIG. 3. Log rate of inversion of sucrose by invertase is plotted against 1/T. The reciprocal of the time required to liberate one mg. invert sugar is taken as a measure of rate.

The data presented (Figs. 1, 2, and 3) indicate that this procedure is not justified.

DISCUSSION

For all three enzymes studied the rate of hydrolysis of the three major classes of foods increases exponentially with temperature in accordance with the Arrhenius equation. For each enzyme there is a sharp break in the relationship at 0 to —2°C. with a relatively low activation energy characterizing the system above this temperature and a relatively high one below this critical temperature. In all cases, except for the lipase digest which contains glycerol, this break in the temperature kinetics occurs at about the transition point from liquid to solid and would seem to be in some way related to this change in state. There is no inactivation of these enzymes by very low temperatures, and the retardation in rate which occurs below —2°C. can be adequately accounted for by the usual exponential relationship of reaction velocity to temperature, with a high activation energy of the enzyme system.

From a practical point of view it is apparent from these studies on lipase, trypsin, and invertase that calculations of enzyme action at low temperatures based on measurements of enzyme action at high temperatures are unreliable owing to the sharp change in the temperature kinetics which occur at about —2°C. The high activation energies for these enzyme systems at low temperatures should be of special interest to those concerned with the storage of foods and other biological materials at low temperatures, since it means that relatively small decreases in temperature below freezing will be relatively very effective in retarding undesirable enzyme action. For lipase, trypsin, and invertase a unit decrease in temperature below —2°C. is roughly five times as effective in retarding enzyme action as a similar decrease in temperature above 0°C. Studies reported in this paper clearly indicate, however, that enzymes are not in any way destroyed by very low temperatures, so that when cold-storage foods are returned to normal temperatures, enzyme action will regain its original rate.

SUMMARY

The kinetics of lipase, trypsin, and invertase action have been studied over the temperature range from 50 to —70°C. For all three enzymes at temperatures both above and below 0°C. the rate of hydrolysis increases exponentially with temperature in accordance with the Arrhenius equation. Heat inactivation of these enzymes occurs above 40°C. A sharp break in the relationship of rate to temperature appears at 0 to —2°C. with a low activation energy characterizing the system above this temperature. The activation energies obtained are as follows: lipase 7,600 cal., 37,000 cal.; trypsin 15,400 cal., 65,000 cal.; invertase 11,100 cal., 60,000 cal. For all the enzyme systems studied (except for lipase with glycerol present in the digest where the freezing point was —18.5°C.) the shift in activation energy from a low to a high value occurred at about the temperature where the digest changed from liquid to solid. In view of this shift in kinetics at this point, it is impossible to predict enzyme action below 0°C. from studies made above this temperature.

Although enzyme action is markedly retarded in the solid state, low temperatures do not inactivate enzymes. The storage of lipase, trypsin, and invertase for 27 days at —70°C. did not affect their hydrolytic activity when subsequently measured at 30°C. The application of these results to the problem of low-temperature storage of foods is pointed out.

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IRON CONTENT OF POTATOES AS INFLUENCED BY COOKING METHOD

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The present investigation was undertaken to determine possible differences in the iron content of a given variety of potatoes when boiled, steamed, or baked. The Smooth Rural potatoes used in all experiments were grown from one seed lot in the season of 1938, in Tompkins County, New York, on a medium loam soil which had been given the same fertilizer treatment for five years, namely 800 pounds of 5-8-7[1] fertilizer and 10 tons of manure per acre. After being harvested in October, the potatoes were stored at 4.4°C.(40°F.) but were removed to room temperature seven to 10 days before cooking. The average specific gravity of the potatoes was 1.082; the weight varied from 125 to 250 grams. The experimental period extended from February to May, 1939.

EXPERIMENTAL PROCEDURE

Methods of Preparation and Cooking: Potatoes for boiling and steaming were washed and either left unpared or were pared with a patented slot-type of potato parer, used in such a way as to give paring losses of about 12 per cent. After being pared, the potatoes were rinsed quickly in tap water. For boiling, five-quart, heavily enamelled, iron pans were used; they were fitted with lids that had been heavily coated with tin and cut to hold a cork and a thermometer. The tap water used was iron-free even after it was boiled 30 minutes in these pans. It had a pH of 7 to 8 and contained 40 to 100 parts per million of calcium carbonate. The potatoes were placed in boiling water except when they had been soaked prior to cooking, in which case they were started in the water in which they had been soaked. Twice as much water by weight as potato was used. The thermometer was inserted in one of the tubers. For steaming, the potatoes were placed in an aluminum steamer, the lower compartment of which contained actively boiling water. For baking, an uninsulated gas oven regulated at approximately 232°C. (450°F.) was used. During both steaming and baking a thermocouple was inserted in one of the tubers.

Five potatoes of approximately equal weight and shape were cooked at one time. The total weight of the raw and of the cooked tubers, including sloughed residue if any, was recorded to the nearest gram. One or two tubers from each group were selected for iron determinations; the remainder were used for other tests. The potato that held the thermocouple or thermometer was not used for analysis. All whole tubers were cooked to an internal temperature of 97.8°C.(208°F.) since previous experiments in this

[1] Five per cent nitrogen (N), eight per cent phosphorus (P), and seven per cent potassium (K).

laboratory had indicated that at this temperature potatoes are satisfactorily cooked. Quarters were cooked for 20 minutes.

In some cases the potatoes were boiled in salted water containing two teaspoons of salt in each quart of water. Three types of commercial salt were used: one had no filler, the second was similar to the first but contained one per cent by weight of calcium carbonate filler, and the third was similar to the first but contained one per cent by weight of tricalcium phosphate filler.[2] All salts and fillers were analyzed for iron.

Methods of Sampling: Cooked potato—If cooked unpared, the skin was removed; the potato was cut into quarters and two diagonally opposite quarters were thoroughly mixed in a porcelain mortar. Two samples of .8 to 1.5 grams were transferred to the 50-ml. volumetric flasks used for the complete analytical procedure.

Raw potato—Two thin slices were cut through the center of the entire pared potato, one cut being made at right angles to the other. The slices were ground in a mortar and two samples were taken and weighed as for the cooked tubers.

Potato skin—Small sections of the skins of boiled potatoes and of those portions of the baked potatoes that had not been in contact with the iron rack in the oven, were selected for iron determinations. The skins of the boiled potatoes were thin and free from potato flesh, but the skins of the baked potatoes were crisp and heavy with portions of the cortex baked on and clinging to them. The material was analyzed as was the cooked potato flesh.

Cooking water—Duplicate samples of approximately 10 ml. of unfiltered cooking water were pipetted into 50-ml. volumetric flasks and analyzed.

Determination of Total Iron: The procedure for the determination of total iron was a slight modification of the o-phenanthroline method suggested by Sheets (1938), which is an adaptation of the method developed by Saywell and Cunningham (1937). After wet ashing with sulfuric acid, digestion with perchloric acid, and the addition of hydroxylamine and o-phenanthroline, the solution was partially neutralized with ammonium hydroxide and made up to volume. With each series of determinations, a blank consisting of the reagents was included. The blanks for each set of reagents were comparatively constant and very low, thus indicating no iron contamination from external sources. A Cenco-Sanford Sheard photelometer was used for measuring the intensity of the colored complex. The results were reported as milligrams of iron per 100 grams of potato.

At least 10 individual tubers were analyzed and the results averaged in order to secure a representative value for a given group of tubers. Duplicate samples were taken and the values averaged and used only when the agreement was within .09 milligram of iron per hundred grams of potato. For most determinations, however, the agreement was considerably closer.

Determination of Ionizable Iron: In order to determine the relationship between the total and ionizable iron in the potatoes, some samples were treated concurrently by a second procedure, which was a modification of

[2] All three salts were prepared and supplied by the International Salt Co., Inc., Ithaca, N. Y.

that of Elvehjem, Hart, and Sherman (1933) and Sherman, Elvehjem, and Hart (1934). The samples were weighed to the nearest milligram in rubber-stoppered, 50-ml. centrifuge tubes; 10 ml. of distilled water and one drop of concentrated sulfuric acid were added to each. The addition of sufficient ammonium hydroxide to turn Congo-red paper red was followed by the addition of one ml. of hydroxylamine hydrochloride and one ml. of o-phenanthroline. When the solution turned pink, five ml. of 95-per cent ethyl alcohol were added. After standing overnight the mixtures were centrifuged until clear and the resulting solution read as previously described. A number of the samples were rendered worthless by a turbid appearance after centrifuging. The absorption of considerable pink color by raw potatoes offered some difficulty.

Correction for Water Absorption and Water Loss: The difference between the weight of the raw and cooked tubers, including sloughed residue if any, divided by the weight of the raw tuber and multiplied by 100 was considered to be the percentage of water absorbed or lost. Previous work in this laboratory had indicated that when a correction was made for the small amount of material dissolved in the cooking water, the water-absorption percentages were not affected significantly. The average per cent of water absorbed or lost during cooking was obtained for each lot of cooked potatoes. In order to obtain the net change in water content, however, this value was corrected for the loss of moisture that occurred during sampling. This additional correction was derived from several lots of potatoes cooked by the different methods. After the lot had been weighed, several samples were thoroughly mixed in mortars, in a manner similar to that used in sampling, and reweighed; the difference in weight constituted water lost during sampling. By this means it was found that the net changes for the Smooth Rural potatoes used were as follows: steamed, pared and boiled, unpared potatoes, average net loss two per cent; whole pared, boiled potatoes, average net gain four per cent; quarters of pared, boiled potatoes, average net gain two per cent; whole pared tubers soaked previous to boiling, average net gain six per cent; baked potatoes, average net loss 24 per cent.

Calculation to Raw Basis: If, during cooking and sampling, the net gain of water was "b" per cent, a determined value of "a" milligrams of iron per 100 grams of cooked potato became a $\left(1 + \frac{b}{100}\right)$ milligrams of iron per 100 grams when calculated to the raw basis. If water was lost, calculation to the raw basis was made by a $\left(1 - \frac{b}{100}\right)$.

DATA AND DISCUSSION

The standard error of the mean for the groups of 10 potatoes studied was usually no greater than $\pm$ .03 milligram of iron per 100 grams of potato. The iron content did not vary with the weight of the potato. There was no significant difference between the total iron content of the flesh of raw potatoes, of potatoes steamed after paring, and of those boiled whole but peeled after cooking (Table 1). The paring loss of raw potatoes aver-

aged only 12 per cent and included a small amount of cortex. No cortex was attached to the thin skin that was peeled from boiled potatoes. Thus, significant quantities of iron were not lost when whole potatoes were boiled unpared, or when they were thinly pared and then steamed. In studies of the total mineral losses on the boiling of unpared potatoes, Snyder (1897) reported only a trifling loss, and Griebel and Miermeister (1926) reported a loss of six to seven per cent. The iron content determined on the cooked basis was not very different from that calculated on the raw basis, because the net change in water content attributable to absorption during cooking and loss during sampling was small. This was equally true of potatoes boiled after paring, even though the net water absorption sometimes reached an average of six per cent.

TABLE 1

Effect of Method of Cooking on Iron Content of Smooth Rural Potatoes

Method of preparation and cooking	Number of potatoes	Average iron content	
		Cooked basis	Raw basis
Raw		*mg./100 gm.*	*mg./100 gm.*
Pared before testing	10	.....	.81 ± .028[1]
Steamed			
Pared before cooking	11	.82 ± .020	.80
Boiled			
Unpared; skin removed after cooking	10	.78 ± .027	.76
Pared; started in boiling water	14	.69 ± .033	.72
Pared; cut in quarters and started in boiling water	10	.72 ± .019	.73
Pared; cooked whole, soaked six hours, started in cold water	10	.69 ± .029	.73
Baked			
Thick skin removed after baking	10	.89 ± .023	.68

[1] Standard error of the mean $S_{\bar{x}} = \frac{S}{\sqrt{N}}$.

Similar quantities of iron were found in pared potatoes that had been boiled whole, boiled in quarters, and soaked whole in water for six hours previous to boiling (Table 1). Approximately the same quantity of iron was also found in pared tubers that had been held either in a dry pan or in the cooking water for 15 minutes after cooking; the iron content was .67 ± .30 and .70 ± .032 milligram per 100 grams of cooked potato, respectively; calculated to the raw basis, the values are .70 and .73 milligram. The iron content of all potatoes pared previous to boiling was significantly lower than that of potatoes boiled unpared or steamed after paring (Table 1). The lower value was not due to a relatively high concentration of iron just under the skin but to the dissolving of iron during boiling, since raw potatoes pared thinly before testing contained the same proportion of iron as boiled potatoes from which the thin skin was removed after cooking. The pared potatoes lost an average of about 10 per cent of their iron during boiling. This value was confirmed by analyses of some of the residual cooking waters. The loss is of the same order of magnitude as is the 15-per cent

loss reported by Blunt and Otis (1917) for whole potatoes pared before boiling. It is considerably smaller, however, than the 36-per cent loss implied by McCance and Widdowson (1940); they found an iron content of .75 milligram per 100 grams of raw potato but only .48 milligram for potatoes boiled after paring. The amount of paring waste is not given. The loss is also considerably smaller than the high losses reported by Stienbarger (1923) but her conclusions were apparently based on very limited data.

The iron content of the baked potatoes was definitely higher than that of the tubers cooked in other ways (Table 1). This was due to a 22-per cent decrease in weight because of loss of moisture during baking and an additional two per cent during sampling, making a total moisture loss of 24 per cent. From a given weight of raw potatoes, however, the quantity of iron obtained after baking and the removal of the thick skin was comparable only to the quantity obtained from an equal weight of potatoes boiled after paring. A high concentration of iron in the outer portion of the potato is thus indicated, although judged by the similarity of the iron content of pared raw potatoes and that of boiled potatoes from which the thin skin was removed after cooking, it seems probable that the high concentration is not immediately beneath the skin. Confirmation of this is being sought by analysis of the different portions of the potato.

For purposes of sampling, the baked-potato flesh was removed in a manner that left approximately the same amount of cortex clinging to the skin as might be left if a baked potato were being prepared at table previous to consumption. Since an oven temperature of 232°C.(450°F.) was used, the skins were brown, crisp, and thick because of the cortex on them. The average weight of such skins from a 200-gram raw tuber was 20 grams. The total iron content of eight such skins varied from 4.9 to 10.8 milligrams per 100 grams and averaged 7.5 milligrams. Thus, approximately one to two milligrams of total iron were contained in the skins of baked potatoes when the raw potatoes had weighed 200 grams. Only one-half of the total iron was found to be ionizable. Whether the figures for the ionizable iron in the baked-potato skins are reasonably accurate is open to question; the procedure used may not have been sufficiently drastic to liberate all of the ionizable iron; also difficulty was encountered in reading the photelometer because of the interfering color from the skins. The results indicate, however, that thick, crisp, baked-potato skins may contain appreciable amounts of ionizable iron but that the quantity may vary considerably.

The skins from six boiled potatoes were removed, sampled, and analyzed for total iron. Determinations of ionizable iron were not made. The total iron content varied tremendously; values were from 17.6 to 91.5 milligrams per 100 grams of skin, the average being 56 milligrams. It would thus appear that the highest concentration of iron in baked-potato skins is in the thin skin itself but there is evidence of great variation.

The ionizable iron content of the limited number of cooked potatoes that were analyzed was similar to the total iron content (Table 2). This is in accord with the results of Shackleton and McCance (1936), Smith and Otis (1937), and McCance (1939), who found that 93 to 100 per cent of the total

iron of potatoes was ionizable. It is beyond the scope of this work to suggest how much of this iron is available for haemoglobin formation.

TABLE 2

Total and Ionizable Iron Content of Smooth Rural Potatoes Cooked by Several Methods

Method of cooking	Number of potatoes	Average iron content	
		Total	Ionizable
		mg./100 gm.	*mg./100 gm.*
Steamed, pared	7	.85 ± .020	.82 ± .043
Boiled, unpared	5	.78 ± .043	.79 ± .057
Boiled, pared	8	.69 ± .043	.65 ± .043

TABLE 3

Iron Content of Smooth Rural Potatoes Cooked in Unsalted and in Salted Boiling Water After Paring

Whole or pieces	Number of potatoes	Kind of salt used	Average iron content	
			Cooked basis	Raw basis
			mg./100 gm.	*mg./100 gm.*
Whole	14	No salt	.69 ± .033[1]	.72
Whole	12	Salt without filler	.74 ± .026	.76
Whole	11	Salt with $CaCO_3$ filler	.68 ± .033	.70
Quarters	10	No salt	.72 ± .019	.73
Quarters	10	Salt without filler	.72 ± .019	.73
Quarters	10	Salt with $CaCO_3$ filler	.71 ± .027	.72

[1] Standard error of the mean $S_{\bar{x}} = \frac{S.}{\sqrt{N}}$

The addition of salt to the water in which pared potatoes were boiled had no significant effect on the quantity of iron lost (Table 3). The values obtained when salt with a phosphate filler was used were abnormally high—.86 milligram of iron per 100 grams of cooked potato and .83 milligram calculated to the raw basis. They are not given in the table. Since the presence of phosphate in more than very small traces was shown by Fortune and Mellon (1938) to give a color with o-phenanthroline, the somewhat high values may be attributed to the phosphate in the filler rather than to the use of the salt as such. Confirmation of this assumption was obtained by analyzing iron solutions to which salt with phosphate filler had been added in the concentration used in the cooking water. The values for the net iron content were greater than the sum of the values of the standard solution and the iron content of 6.3 parts per million found for the salt. As the concentration of the standard iron solution was increased, the presence of the salt caused even greater discrepancies.

According to Fortune and Mellon (1938) calcium and carbonate ions do not interfere with the o-phenanthroline method for iron. When either salt without filler or salt with calcium-carbonate filler was added to standard

iron solutions, the value for iron obtained was the sum of the iron present in the standard solution and that in the salt, regardless of the concentration of the standard solution. The salt containing no filler had an iron content of 2.5 parts per million as compared with 5.7 parts per million for the salt with the calcium-carbonate filler, the difference being due to the presence of iron in the filler. In the salt solutions used for cooking, iron was present in a concentration of .03 part per million if the salt contained no filler and .07 part per million if the salt contained calcium carbonate filler. The presence of this amont of iron had no observable effect on the iron content of pared potatoes cooked in the water.

That the addition of salt to the cooking water did not affect the quantity of iron lost when pared Smooth Rural potatoes were boiled, is of interest in view of other work on the mineral losses of boiled vegetables. Lanman and Minton (1927) found a greater loss in the total ash of several vegetables when salt had been added to the cooking water. Stienbarger (1923) noted an increase in the loss of iron when potatoes and other vegetables were cooked in salted water, but her data were limited.

SUMMARY

The total iron content of raw New York State Smooth Rural potatoes grown under known cultural conditions was determined by the orthophenanthroline method and compared with the values obtained for potatoes cooked by different methods. Determinations of ionizable iron were made on some of the potatoes and some of the baked-potato skins.

For these potatoes there was no significant difference between the total iron content of the flesh of raw potatoes, of potatoes steamed after paring, and of those boiled whole but peeled after cooking. The iron content of potatoes so prepared was, however, significantly greater than the iron content of pared potatoes that had been boiled whole, boiled in quarters, or soaked whole for six hours previous to boiling. Potatoes cooked by these methods contained similar quantities of iron; they lost an average of approximately 10 per cent of their iron during boiling. Although the total iron content of baked potatoes removed from their thick skins was relatively high, because of loss of moisture during baking, recalculation to the raw basis gave values comparable to those of potatoes that had been boiled after paring. High concentrations of iron were found in the thick, crusty skins of baked potatoes. The total iron content and ionizable iron content of potatoes were found to be similar. The addition of salt to the water in which pared potatoes were boiled had no significant effect on the quantity of iron lost.

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LOSSES OF VITAMIN C DURING COOKING OF PARSNIPS[1]

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The ascorbic acid content of both raw and cooked parsnips, as reported in the literature, shows a great variation—from five to 40 milligrams per 100 grams of raw parsnip and from an increase of 22 per cent to a loss of 36 per cent owing to cooking. McHenry and Graham (1935) reported 3.7 mg. of reduced ascorbic acid per 100 gm. of raw parsnip, 5 mg. per 100 gm. after treatment with hydrogen sulfide, and 6.1 mg. per 100 gm. of parsnips boiled 30 minutes. This is an increase of 22 per cent upon cooking. Tauber and Kleiner (1935) found 36 to 40 mg. of ascorbic acid per 100 gm. of raw parsnip. Lunde (1938) attributed a value of 20 mg. per 100 gm. to Norwegian-grown parsnips, while Mathiesen and Aschehoug (1937) reported 16 to 13 mg. per 100 gm. in raw and canned parsnips, respectively; that is, a loss of 19 per cent in canning. The above work was done on unnamed varieties.

Wheeler, Tressler, and King (1939) reported 40 mg. per 100 gm. in November in freshly dug parsnips of the Bloomsdale variety. Their parsnips, which were stored in a pit during the winter, decreased to 14.9 mg. per 100 gm. by March, a loss of 63 per cent. Burrell and Ebright (1940) found that garden-fresh parsnips of the Hollow Crown variety had a vitamin C content of 32.6 mg. per 100 gm.

Mayfield and Richardson (1940) reported from 12.8 to 40 mg. of ascorbic acid per 100 gm. of raw parsnip of the Hollow Crown variety in the fall and from 5.5 to 12.7 mg. per 100 gm. of raw parsnip in the spring when the parsnips had been stored in the ground all winter. This was a loss of 60 to 70 per cent. The same authors also reported a loss of 13 per cent of the original ascorbic acid, in the fall, when the parsnips were boiled whole with the skins on, and a loss of 36 per cent when the parsnips were peeled, sliced lengthwise, and boiled. In the spring they found "very little loss when parsnips were boiled whole, but about 26 per cent loss when the parsnips were peeled and sliced before boiling."

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This paper is taken from a thesis to be submitted by E. Josephine Brown to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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These workers purchased their parsnips "at the local market in the fall and in the spring, soon after being dug." Only one parsnip was cooked at a time and the cooking periods seem rather long for this vegetable—30 minutes for boiling the whole, unpeeled parsnips and 20 to 25 minutes for boiling the peeled, sliced parsnips. No distinction in either of the cooking studies was made between the loss of vitamin C owing to solution and that owing to actual destruction. In most of the above reports no analyses for dehydroascorbic acid were given.

The purpose of this study was to determine the vitamin C content of raw parsnips, the effect of the various methods of cooking in amounts used by the housewife, the effect of the composition of the cooking utensil upon the vitamin C content, and incidentally, the losses of vitamin C during storage.

EXPERIMENTAL PROCEDURE

Parsnips of the Hollow Crown variety were grown in the University gardens near the campus on Dunkirk sandy loam soil to which 1,200 pounds of 5-10-5 fertilizer per acre had been added. They were planted in June and dug in November, at which time they were sorted as to size. Those of medium size, chosen for this study, were stored immediately in a constant temperature room held at 1.1 to 1.7°C.(34 to 35°F.) and at a humidity of 95 to 100 per cent. On the days when the vitamin C determinations were to be made, some of the parsnips were transferred to the hydrator tray of an electric refrigerator held at 34 to 35°F.

Ascorbic acid and dehydroascorbic acid determinations were made on the raw parsnips, the cooked parsnips, and the cooking water. From the results of these determinations it was possible to calculate the per cent retention, the per cent solution, and the per cent destruction of vitamin C during the various methods of cooking. From the values obtained for the raw parsnips it was possible to study the effect of storage upon the vitamin C content of the vegetable. The ascorbic acid content of the various parts of the parsnip was determined.

Chemical Determinations: The vitamin C determinations were made according to the Bessey and King (1933) chemical-titration method, as modified by Mack and Tressler (1937). The parsnips were extracted with a freshly prepared solution of eight per cent of acetic acid and two per cent metaphosphoric acid. Duplicate 10-gm. samples of both the raw and cooked parsnips were used. Four extractions were made for each sample using 30, 30, 20, and 20 ml. of extractive, and the vegetable extract was made up to 100 ml. Twenty-milliliter aliquots of the extracts were used for titration. Aliquots of the extracts were saturated with hydrogen sulfide in order to reduce any dehydroascorbic acid to ascorbic acid. The method was essentially that of Tillmans, Hirsch, and Jackisch (1932).

Two 25-ml. aliquots of the cooking water were added to two 20-ml. portions of chilled extractive in two 50-ml. volumetric flasks and the flasks were made up to volume with the extractive. The entire samples were then titrated for vitamin C.

Method of Sampling Raw Parsnips: The raw samples were radial sections from the entire length of each parsnip. The radial-section method

of sampling, which includes a representative portion of each part of the parsnip, was necessary because of the great variability in the ascorbic acid content of the different parts of the parsnip. In each sample, radial sections of four parsnips were used. This number seemed advisable because of the great difference in the vitamin C content of the individual parsnips. In the cooking tests of whole, unpeeled parsnips radial sections were taken from each of the four parsnips cooked. The cut surface exposed to the water or steam was probably as small in this method of sampling as in any other.

Cooking Methods: With the exception of the studies in which four medium-sized, whole parsnips were used, 300 gm. (E.P.) or four servings of parsnips were cooked, using methods and utensils comparable to those normally used by the housewife. The parsnips were cooked by boiling, by steaming, and in two makes of pressure saucepans. The parsnips were cooked (1) whole, unpeeled; (2) peeled, cut crosswise, the upper part quartered, the lower part halved; and (3) peeled and sliced in ⅛ to ¼ inch slices. Throughout the remainder of this article Method 2 will be termed "pieces." These pieces are the size and shape commonly used for deep-fat frying.

For the studies on boiling, the salted tap water was boiling vigorously when the weighed parsnips were added and the heat was so adjusted that the water came back to the boil within two minutes. The rate of evaporation during the cooking was controlled by means of a gas plate, manometer, and balance arrangement similar to that described by Fenton, Tressler, Camp, and King (1937).

Four different saucepans were used for the studies on boiling. Each pan had a capacity of two quarts, a diameter of about seven inches and a depth of about four and one-half inches. The saucepans were made of enamel, Pyrex, stainless steel, and aluminum.

For the steaming of parsnips, water was placed in the lower compartment of the steamer, the upper part and cover were set in position, and the water was brought to boiling so that both compartments were filled with steam before the parsnips were added. The steamer used was made of aluminum and had a perforated bottom in the upper compartment.

The pressure saucepans used were the Presto and the Flex Seal. Both were made of aluminum and each had a capacity of two quarts. To check the temperatures reached by each of these pressure saucepans some special devices were used. The Presto cooker was equipped with a pressure gage. A thermometer was inserted in place of the small rubber disc in the cover of the Flex Seal. The temperatures reached in the cookers were also checked with a maximum temperature thermometer. The cover or weight was placed on the vent each time when the temperature reached about 98.9°C.(210°F.), which was the boiling temperature of tap water in the laboratory. This meant that in each test most of the air had been expelled and it was possible to attain a temperature of 119.4°C.(247°F.), when the indicator on each registered 15 pounds of pressure. To lower the pressure when the cooking was finished each pan was set immediately into cold water, the top valve was opened, and the cover removed. The

parsnips were removed from the pressure saucepans within one minute from the time the pan was taken from the heat.

DISCUSSION OF RESULTS

During November, December, and January the vitamin C content of individual parsnips varied from 15 to 30 mg. per 100 gm. of raw parsnips and the range remained about the same during the entire period. However, during February and early March the values decreased to a range of seven to 18 mg. per 100 gm. The range of values during each period was independent of the size of the parsnips used. The amount of dehydroascorbic acid present varied from nothing to 25 per cent. There seemed to be no consistent relationship between the amount of dehydroascorbic acid and the length of the storage period. Determinations of the vitamin C content of the different parts of the parsnip at intervals during storage

TABLE 1

Effect of Method of Cutting Upon Vitamin C[1] Content of Boiled and Steamed Parsnips

Method of cutting	Number of experiments	Cooking time[2]	Vitamin C				
			Raw parsnips	Cooked parsnips	Boiled parsnips[3]		
					Retention	Solution	Destruction
Boiled [3]		*min.*	*mg./100 gm.*	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
Whole unpeeled [4]	6	15	20	18	91	5	4
Pieces [5]	8	10	22	18	81	10	9
Sliced	4	8	12	10	84	15	1
Steamed [6]					Steamed parsnips[6]		
Whole unpeeled [4]	10	25	17	15	86	....	14
Pieces [5]	9	20	20	17	83	....	17

[1] Ascorbic and dehydroascorbic acid. [2] Cooking time counted from moment parsnips were dropped into pan. [3] Enamel saucepan with 500 ml. water. [4] Radial section removed for raw samples. [5] Peeled, cut crosswise, upper part quartered, lower part halved. [6] Aluminum steamer with perforations in bottom of upper compartment.

showed that the relative distribution of vitamin C varied during the storage period. In the fall the tip contained twice as much vitamin C as did the upper portion, and the medulla contained one and one-half times the amount in the cortex. This distribution of vitamin C was not maintained throughout the storage period. To overcome these variations it was necessary in some cases to make as many as eight determinations and to use the radial section for sampling.

The effect of the method of cutting upon the vitamin C retention of boiled and steamed parsnips is shown (Table 1). When boiled, uncovered, in 500 ml. of salted tap water, whole, unpeeled parsnips retained 91 per cent of their ascorbic acid; peeled parsnips, cut in pieces, 81 per cent; and peeled, sliced parsnips, 84 per cent. Although the cut surface was greater when the parsnips were sliced before boiling, the shorter cooking time required probably accounts for the smaller loss of vitamin C in this method than the loss when the parsnips were cut in pieces. The ascorbic acid-oxidizing enzyme was probably inactivated sooner in the smaller pieces.

Parsnips that were steamed whole (except for the removal of the radial section) and unpeeled, retained 86 per cent of the vitamin C, while those cut in pieces and steamed retained 83 per cent.

The figures (Table 2) show the effect of the composition of the saucepans upon the vitamin C content of parsnips cooked by boiling. Sliced parsnips boiled, uncovered, in 500 ml. of salted tap water in saucepans of nearly identical size, made of enamel, Pyrex, stainless steel, and aluminum showed vitamin C retentions of 84, 81, 66, and 71 per cent, respectively. In the case of the enamel and Pyrex saucepans most of the vitamin C lost from the parsnips was found in the cooking water. This was not

TABLE 2

Effect of Composition of Saucepans Upon Vitamin C[1] Content of Parsnips[2] Cooked by Boiling[3]

Composition of pan	Number of experiments	Vitamin C				
		Raw parsnips	Cooked parsnips	Boiled parsnips		
				Retention	Solution	Destruction
		mg./100 gm.	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
Enamel	4	12	10	84	15	1
Pyrex	5	18	15	81	19	0
Stainless steel	6	17	11	66	10	24
Aluminum	7	14	10	71	16	13

[1] Ascorbic and dehydroascorbic acid. [2] Parsnips sliced crosswise. [3] Boiled in 500 ml. of salted tap water for eight minutes, that is time counted from moment parsnips were dropped into boiling water.

true with the stainless steel and aluminum saucepans; in them some actual destruction occurred. McIntosh, Tressler, and Fenton (1942) reported that the relative effect of the composition of the cooking utensil varies with the kind of vegetable and that the kind of vegetable may be a more important factor than the composition of the pan. Floyd and Fraps (1940) reported some variation in the vitamin C losses when 50 gm. of turnip greens were boiled rapidly in uncovered utensils of different compositions. These losses were 18.3, 17.1, 15.5, and 26.1 per cent for stainless steel, Pyrex, enamel, and aluminum, respectively. When the pans were covered, the losses were nearly the same, 26.2, 24.2, 26.7, and 26.9 per cent, for stainless steel, Pyrex, enamel, and aluminum, respectively. This is in agreement with the work of Barnes, Fenton, and Tressler (1942) who found no difference in the percentage retention of vitamin C in quick-frozen broccoli cooked in pans of these same materials.

When parsnips cut in pieces were boiled in 500 ml. and 750 ml. of salted tap water, about the same vitamin C retention was obtained, 81 and 83 per cent, respectively (Table 3).

When parsnips cut in pieces were boiled in 500 ml. of water in a covered enamel pan, the ascorbic acid retention was a little higher (Table 6) than when the pan was uncovered, 86 versus 81 per cent. The slightly greater retention of vitamin C in cooking in the covered pan may have been caused by the water returning to the boil sooner.

Pressure saucepans were also used for cooking parsnips. This method of cooking gave a high retention of vitamin C; pieces cooked to doneness in two minutes at 15 pounds pressure retained 90 per cent of their vitamin C. Underdone parsnips cooked one minute at 15 pounds pressure retained 97 per cent, and overdone parsnips held five minutes at 15 pounds pressure retained 78 per cent of their vitamin C (Table 4). The last value

TABLE 3

Effect of Varying Volume of Water Upon Vitamin C[1] Content of Boiled Parsnips[2]

Volume of water used	Cooking time[3]	Number of experiments	Vitamin C				
			Raw parsnips	Cooked parsnips	Boiled parsnips[2]		
					Retention	Solution	Destruction
ml.	*min.*		*mg./100 gm.*	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
500	10	8	22	18	81	10	9
750	10	8	24	20	83	8	9

[1] Ascorbic and dehydroascorbic acid. [2] Peeled, halved crosswise, the upper part quartered, the lower part halved. [3] Cooking time counted from time parsnips were placed in boiling water.

shows the need for avoiding overcooking with a pressure saucepan. The lower result obtained for parsnips cooked five minutes at 15 pounds pressure may be at least partially explained on the basis that it was not possible to exclude all of the air from the pan before the small weight was put over the vent. It cannot be explained by an increase in moisture content since the calculation of results included the weight of the parsnips before and after cooking, thus eliminating the effect of such an increase. Although most of these determinations were done while using the Flex Seal pressure saucepan, results of a study in which sliced parsnips were

TABLE 4

Effect of Time Upon Vitamin C[1] Content of Parsnips[2] Cooked in Flex Seal Cooker

Time held at 15-lb. pressure	Number of experiments	Vitamin C				
		Raw parsnips	Cooked parsnips	Cooked parsnips		
				Retention	Solution	Destruction
min.		*mg./100 gm.*	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
1 (underdone)	2	20	19	97	2	1
2 (done)	4	25	23	90	4	6
5 (overdone)	2	21	16	78	2	20

[1] Ascorbic and dehydroascorbic acid. [2] Parsnips peeled, halved crosswise, the upper part quartered, the lower part halved.

cooked two minutes at 15 pounds pressure in both the Flex Seal and the Presto pressure saucepans (Table 5) showed 86 and 83 per cent vitamin C retention, respectively. The difference between the two values is within the error of the method. Although the best vitamin C retention in the parsnips was obtained by cooking them in the pressure saucepans, the vegetable was not as uniformly done as when cooked by the other methods. The xylem (inner portion) was underdone and somewhat stringy when

the phloem (outer portion) was soft. Simpson and Halliday (1941) report that the cell walls of the xylem tissues of raw parsnips "were considerably thicker and more continuous than those of either the cambium or the phloem" and "it requires slightly longer for the inner (xylem) portion of the root to become tender during cooking than for the outer (phloem) portion."

TABLE 5

Comparison of Vitamin C[1] Content of Sliced Parsnips Cooked in Two Makes of Pressure Saucepans

Pressure saucepan[2]	Number of experiments	Vitamin C				
		Raw parsnips	Cooked parsnips	Cooked parsnips[3]		
				Retention	Solution	Destruction
		mg./100 gm.	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
Flex Seal	4	19	16	86	9	5
Presto	5	20	17	83	12	5

[1] Ascorbic and dehydroascorbic acid. [2] Both pressure saucepans were heavy aluminum, two-quart capacity. [3] Held two minutes at 15 pounds pressure.

The results (Table 6) indicate the average changes in vitamin C content of parsnips cooked by different methods. Parsnips, cut in pieces, were boiled, uncovered and covered, in an enamel saucepan, in 500 ml. of salted tap water. They were also steamed and cooked in pressure saucepans. They retained 81 per cent of their vitamin C when boiled uncovered, 86

TABLE 6

Average Change in Vitamin C[1] Content of Parsnips Cooked by Different Methods

Method of cooking[2]	Number of experiments	Type of pan	Cooking time[3]	Vitamin C				
				Raw parsnips	Cooked parsnips	Cooked parsnips		
						Retention	Solution	Destruction
			min.	*mg./100 gm.*	*mg./100 gm.*	*pct.*	*pct.*	*pct.*
Boiling uncovered	8	Enamel saucepan	10	22	18	81	10	9
Boiling covered	4	Enamel saucepan	10	19	16	86	9	5
Steaming	9	Aluminum steamer	20	20	17	83	17	
Pressure saucepan	4	Aluminum Flex seal	2	25	23	90	4	6

[1] Ascorbic and dehydroascorbic acid. [2] Peeled, cut crosswise, the upper part quartered, the lower part halved. [3] Cooking time for the pressure saucepan was time held at 15 pounds pressure.

per cent when boiled covered, 83 per cent when steamed, and 90 per cent when cooked in pressure saucepans. The retention of vitamin C in boiling was higher than that reported by Mayfield and Richardson (1940). This may be at least partially explained by the shorter cooking times required for both the whole and the sliced parsnips.

SUMMARY

Parsnips, whole and unpeeled; peeled and cut in pieces; and peeled and sliced crosswise were cooked by boiling, by steaming, and in two makes of pressure saucepans. Vitamin C determinations were made on the raw and cooked parsnips and on the cooking water.

The values for the raw parsnips showed that during the months of November, December, and January carefully stored parsnips contained from 15 to 30 mg. of vitamin C per 100 gm. of raw parsnips. During February and early March parsnips contained from seven to 18 mg. of vitamin C per 100 gm. of raw parsnip. The variation in vitamin C content from fall to spring indicates a need for reporting the season of the year when the vitamin C determinations are made.

The relative distribution of vitamin C in the different parts of the parsnip varied during the storage period.

Parsnips boiled whole, unpeeled, and parsnips cut in pieces and cooked in the pressure saucepan gave the best vitamin C retentions of the methods used, 91 and 90 per cent, respectively.

Parsnips steamed whole, unpeeled, and parsnips cut in pieces and boiled retained 86 per cent of their original vitamin C. In both boiling and steaming the parsnips cooked whole, unpeeled, retained more vitamin C than did those which had been sliced or cut in pieces.

Parsnips boiled in enamel and Pyrex pans retained more vitamin C than those boiled in stainless steel and aluminum pans, 84 and 81 per cent, against 66 and 71 per cent, respectively.

Parsnips cut in pieces cooked five minutes (overdone) in the pressure saucepan showed a retention of only 78 per cent of the original vitamin C, whereas the retention was 90 per cent at the done state after two minutes' cooking.

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BLACKENING OF COOKED POTATOES—PROPERTIES OF THE PIGMENT

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The stem blackening of cooked potatoes has been described by Nutting and Pfund (1942) in another report from this laboratory. The data indicate that the blackening process does not involve the formation of melanin. In order to investigate further the nature of the blackening it was considered advisable to study the properties of the pigment involved and to compare them with those of melanin obtained from raw potatoes.

EXPERIMENTAL PROCEDURE

Preparation of Melanin: Melanin was prepared from New York State Smooth Rural and Chippewa potatoes by two processes. One preparation was separated mechanically from the water extracts of raw potatoes. The raw tubers were cut into small pieces, mixed with distilled water, and reduced to a pulp in a Waring Mixer. The fiber was removed from the mixture by straining through a double thickness of cheesecloth. The starch was allowed to settle on standing for half an hour at room temperature. The red supernatant liquid was decanted off and placed in the refrigerator for two days. At the end of that period the solution had blackened and more starch and fiber had settled out. The supernatant liquid was siphoned off and centrifuged eight successive times. The precipitates from the first six treatments in the centrifuge were combined and some solubility tests were made. The last two residues were kept separate and used for spectral analysis. To remove any starch which might have settled out with the melanin an aqueous solution of taka-diastase was added to the precipitates and the mixtures were allowed to stand at room temperature for 24 hours. After several washings with distilled water the melanin was separated by suction filtration; washed with dilute hydrochloric acid, water, absolute alcohol, dilute alcohol, and water; and dried in a vacuum dessicator over sulfuric acid.

A second preparation of melanin was made by the tyrosine-tyrosinase reaction described by Raper and Wormall (1923). In the course of the usual procedure in the attempt to remove the proteins the melanin separated out at the same time and thus was not in a pure form. To prevent contamination by proteins, the tyrosine-tyrosinase reaction was speeded up by the use of a potassium acid phthalate buffer of about pH 6.5. When the buffered solution of tyrosine was added to the raw-potato juice which had been heated to 37°C.(98.6°F.) the mixture turned black immediately. The mixture was heated for five hours at 37°C. The melanin which formed was separated from colloidal solution by the centrifuge and treated exactly as was the melanin obtained from the potato juice alone.

Separation of Pigments From Cooked Potatoes: Blackened portions, which had been cut from discolored, cooked potatoes, were mashed thoroughly in a large mortar. Certain preliminary tests were made on some of this dark gray material: its solubility in water was noted; its behavior with dilute acids, with alkalis, and with organic solvents was observed. Some portions from the cortex around the stem end of cooked, non-blackening tubers were treated similarly. The preliminary tests indicated that in order to avoid the destruction of the blackened material as such it was necessary to work with approximately neutral solutions. Therefore, a method was used by which the pigments could be separated from water solutions by chromatographic absorption.

The potato mash was mixed thoroughly with successive portions of distilled water. The total weight of water used was 10 times the weight of the potato. After the mixture had been stirred well, it was placed in the refrigerator for several days. The fiber was separated by filtration. The liquid was concentrated under reduced pressure at a temperature of 60 to 65°C.(140 to 149°F.) to one-tenth the original volume. After the solution had stood for approximately one week in the refrigerator, the sediment then present was removed by centrifuging.

The preparation of the chromatographs from the extracts obtained from both the blackened and non-blackened potatoes was carried out as described by Zechmeister and von Cholnoky (1937). Glass adsorption tubes, about 15 cm. by 1 to 1.5 cm., were packed to a depth of six to eight centimeters with Merck standardized aluminum oxide. The bottoms of the tubes were plugged with glass wool and covered with cheesecloth. Glass wool was also placed at the top of the columns. Mild suction was applied. The columns were washed either with distilled water alone or with dilute solutions of sulfuric acid followed by distilled water. Approximately 30 ml. of extract diluted with an equal volume of water was allowed to pass through a single column. The exact amount of extract used varied with the intensity of the color of the material. After all the solution had passed through the columns they were washed well with distilled water. Only water alone could be used in the "development" of the chromatographs since other solvents destroyed some of the color. Although rinsing with water did not give as wide separation of the color bands as might have been desired, distinct regions could be observed and separated.

The chromatographs were partially dried, removed from the tubes, and separated carefully into layers. The adsorbed material was kept in a vacuum dessicator until used. Inasmuch as desorption of the pigment affected the color, no dark pigment in a pure form was isolated. However, the effects of various reagents on the adsorbed pigments were observed. The aluminum oxide adsorbent is practically unaffected by the reagents and does not interfere with observations of the color reactions.

Ultra-Violet Absorption Spectra: The ultra-violet absorption spectra of the pigments from blackening and non-blackening tubers were taken. The spectra of the two melanin preparations were observed also. All studies were made on dilute solutions of the material in .05N sodium hydroxide.

A Bausch and Lomb medium quartz spectrograph and a Hilger sector photometer were used to obtain most of the absorption spectra. A Hilger medium quartz spectrograph was substituted for the Bausch and Lomb instrument for part of the work. Eastman Kodak spectrographic plates, Type 103-0, were used. The light source was a four-millimeter spark between tungsten steel electrodes. The solution of the material under examination was placed in one two-centimeter tube, while the other photometer tube was filled with .05N sodium hydroxide solution for the comparison spectrum. The equipment was tested for accuracy by the measurement of the absorption spectrum of a dilute solution of potassium chromate in .05N sodium hydroxide. The absorption curve obtained agreed closely with that given for potassium chromate by Rössler (1921).

Since the molecular weights were all unknown, the percentage extinction coefficients were computed according to Weigert (1927). The percentage extinction coefficients were calculated from the formula $E = \frac{\log I/I_o}{dc}$: where *log* I/I_o was the sector reading, d the length of the tube in centimeters, and c the concentration in grams per hundred milliliters of solution. The absorption curves were plotted using the wave lengths of the match points as abscissae and the logarithm of the extinction coefficients as ordinates.

The melanin solutions were made by dissolving completely small amounts of the two preparations of melanin from filter papers on which they had been carefully washed, dried, and weighed. The solutions of the pigments were prepared by direct desorption of the pigments from the aluminum oxide. The weight of the material in solution was estimated as approximately equivalent to the difference in weight between the adsorbent plus pigment and the dried residue after desorption. The values obtained for the concentrations are undoubtedly too large since the aluminum oxide may give up adsorbed water and also may be partially dissolved in the sodium hydroxide. Neither aluminum oxide nor water would affect the absorption except in respect to the extinction coefficients; they would be too small. This uncertainty in the extinction coefficients does not alter the shape of the absorption curves or the relative positions of the bands, the factors of primary importance in this qualitative work.

DATA AND DISCUSSION

The melanin obtained from raw-potato juice alone and that prepared from potato juice and tyrosine showed the typical characteristics of melanin as described by Jacobsen (1934) and Florence, Enselme, and Pozzi (1935). The brownish-black amorphous material was insoluble in water and organic solvents, unaffected by cold acid solutions, and soluble in dilute alkali. The addition of acid to a brown solution in five per cent sodium hydroxide or potassium hydroxide caused the reprecipitation of the brownish-black melanin. Alkaline peroxide solution destroyed the color and the compound. It is evident, therefore, that the melanin formed from raw tubers is a relatively stable compound.

The ultra-violet absorption spectra of the two melanin preparations (Fig. 1, BI and BII) are identical except for a slight factor in the extinction coefficient. This difference may be due to a slight error in the values for the concentrations because of the small amounts of material used. These spectra are quite similar to the spectrum obtained by Spiegel-Adolf (1937) for a synthetic melanin formed by the action of light on tyrosine and also to that observed by Florence, Enselme, and Pozzi (1935) for a

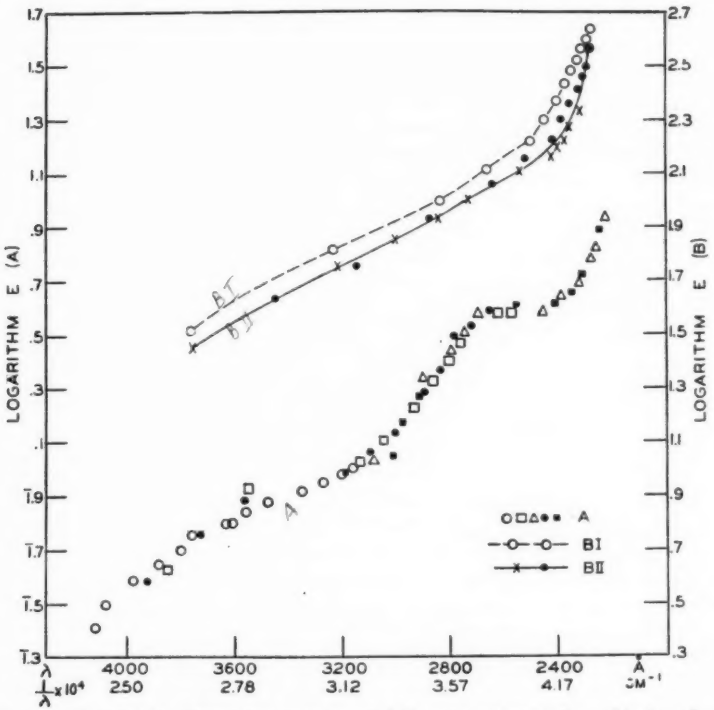

FIG. 1. Ultra-violet absorption spectra of (A) gray pigment from blackened potatoes. ○, c = .393 gm. per 100 ml. .05N NaOH. □ and △, dilutions of ○. ●, c = .130 gm. per 100 ml. ■, dilution of ●; (BI) melanin from raw-potato juice. c = .0015 gm. per 100 ml. .05N NaOH; (BII) melanin from raw-potato juice and tyrosine. ●, c = .0035 gm. per 100 ml. .05 NaOH. ×, dilution of ●.

melanin prepared from tyrosine and tyrosinase. No discrete absorption bands are apparent for any of the preparations of melanin.

The dark color of the potato mash from very dark, blue-black portions of cooked potatoes was removed by washing with large amounts of water. The light green-gray water extract became dark gray after concentration. When dilute acids were added to the potato mash, the blue-black color disappeared completely. The careful addition of sodium hydroxide or potassium hydroxide solution restored the dark color but an excess of alkali resulted in a persistent yellow color. The immediate action of the

alkali was the formation of a yellow color which turned gray when the mixture was agitated. The process could be reversed by the careful addition of acid to the yellow material. A similar yellow color was observed when the mash from non-blackened, cooked tubers was made alkaline. In this case, however, careful acidification gave a transitory brown-gray color rather than a blue-black like that of the blackened tubers. Complete acidification gave a practically colorless material. The concentrated water extracts of both kinds of tubers showed color changes with acid and alkali similar to the behavior of their respective potato mashes.[1]

The addition of ethyl alcohol to the blackened portions removed the dark color and yielded a yellow extract. Apparently the alcohol destroyed the gray color as it could not be restored by any treatment. The alcohol extract was rendered practically colorless in the presence of acids and became yellow again when made alkaline. When the alcoholic extract was evaporated, a bright yellow, somewhat oily precipitate separated. Complete evaporation yielded a glassy yellow residue. The precipitates and residues gave a dark green with ferric chloride and an orange color with uranium acetate. Efforts to crystallize the yellow pigment were fruitless. Yellow alcoholic extracts with identical properties were obtained from non-blackened tubers.

Neither benzene nor ether dissolved the dark pigment of the blackened portions of potatoes.

The chromatographs from the dark gray water extracts had a blue-gray layer at the top few millimeters, then a one-centimeter mixed layer, and below this a bright lemon-yellow band of one to two centimeters. If the column had been washed with sulfuric acid prior to use, the gray layer was wider; whereas the yellow layer was smaller and less intense. Since the aluminum oxide adsorbs hydrogen ions, the resulting increase in alkalinity of the solution upon passage through the column may be responsible for some of the yellow pigment. The dry gray material could be desorbed only by acids or alkalis; the acidic solutions were colorless and the alkaline solutions were brown-yellow. The gray material gave no apparent reaction with uranium acetate or ferric chloride.

The yellow adsorbed pigment showed the flavone-type color reactions as described in the "Official and Tentative Methods of Analysis" of the Association of Official Agricultural Chemists (1940): the yellow pigment formed an intense yellow solution in sodium hydroxide, lost color in dilute acids, and gave a dark gray-green color with ferric chloride solution, a bright yellow color with concentrated hydrochloric acid, and an orange color with uranium acetate solution. A yellow pigment of similar behavior was obtained from the chromatographs prepared from the extracts of non-blackened tubers. These chromatographs showed only a single yellow band about 1.5 centimeters from the top of the adsorption column. No appre-

[1] The relation of the blue-gray color to the hydrogen-ion concentration of the mash or water extract indicated that the pH of the potato itself might be connected with the blackening. Preliminary studies have shown that the stem ends of some tubers have a somewhat higher pH than the rest of the tubers. Further investigation of the relationship between pH and blackening is in progress in this laboratory.

ciable amounts of yellow pigment were observed when the column was pre-treated with sulfuric acid.

The dark pigment of the blackened tubers and melanin are therefore dissimilar as indicated by the solubility of the blue-black material in water and alcohol, the color changes with variation in pH, and the chromatographic work. Moreover, the properties suggest that the pigment is related to the flavones, a class of pigments found in many plants and mentioned by Lowe (1937) as being present in potatoes. The relationship between

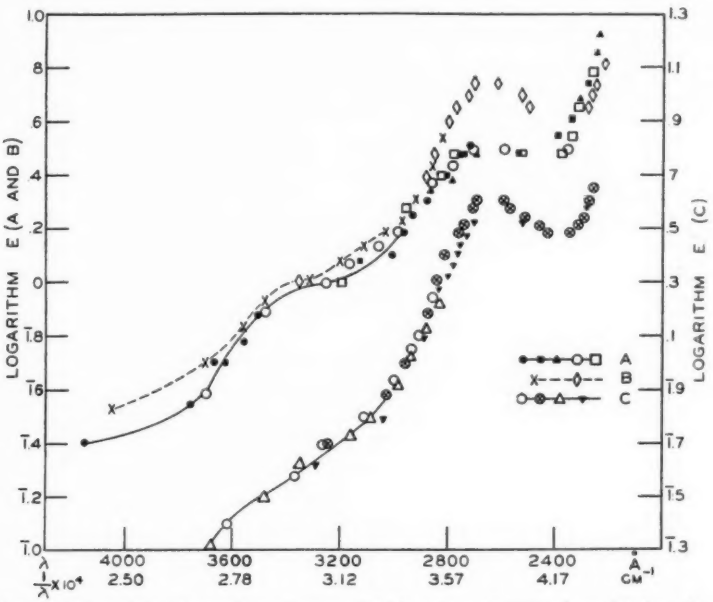

FIG. 2. Ultra-violet absorption spectra of (A) yellow pigment from blackened potatoes (adsorption column pre-treated with H_2SO_4). ●, c = .199 gm. per 100 ml. .05N NaOH. ■, ▲, dilutions of ●. ○, c = .128 gm. per 100 ml. □, dilution of ○; (B) yellow pigment from blackened potatoes (no H_2SO_4 treatment). ×, c = .148 gm. per 100 ml. ◇, × diluted; (C) yellow pigment from non-blackened tubers (no H_2SO_4 treatment). ○, c = .200 gm. per 100 ml. ⊕, dilution of ○. △, c = .240 gm. per 100 ml. ▼, dilution of △.

the yellow pigment of the potato and the blue-black color is not clear. One possibility is that the blue-black color results from the reaction between the yellow pigment and a metal to give a metallic complex similar to the one presumably formed by the yellow pigments and ferric chloride. Wesp and Brode (1934) report that such metallic phenolic complexes are relatively unstable.

The ultra-violet absorption spectrum of the solution of the gray pigment as desorbed in .05N sodium hydroxide (Fig. 1, A) gives further evidence that the material is not a melanin. In order to show the difference between the absorption curve for the gray pigment (Fig. 1, A) and the

continuous absorption of the melanins (Fig. 1. BI and BII), the melanin curves were displaced by using a different set of ordinates. This was necessary since the melanin solutions showed much more intense absorption than the gray pigment. The curve for the gray pigment (Fig. 1, A), however, gives evidence of discrete absorption bands. Two overlapping absorption bands are indicated by the change in slope of the absorption curve between 3,700 Å and 3,100 Å and the plateau between 2,700 Å and 2,400 Å. The maxima in the absorption curve are at approximately 3,450 Å

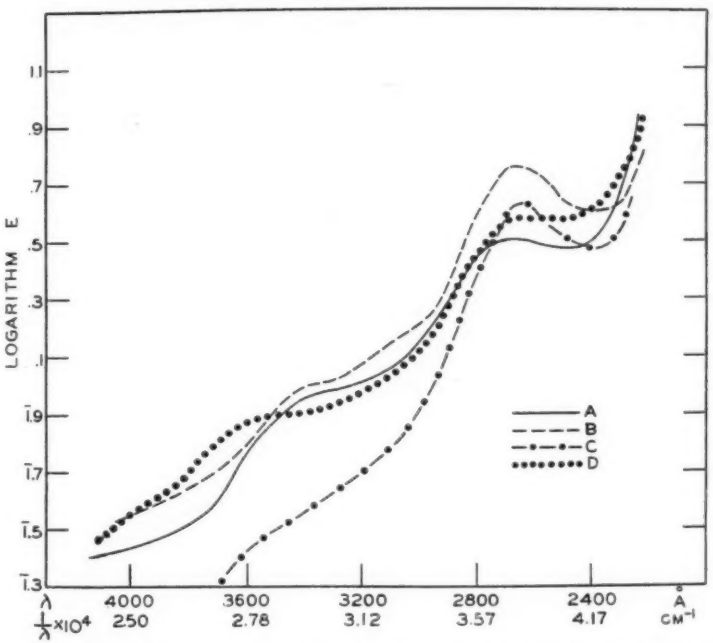

FIG. 3. Ultra-violet absorption curves of (A) yellow pigment from blackened potatoes (column pre-treated with H_2SO_4); (B) yellow pigment from blackened potatoes (no H_2SO_4 treatment); (C) yellow pigment from non-blackened potatoes; (D) gray pigment from blackened potatoes.

and 2,600 Å. No significant difference in the spectrum was apparent when the chromatographic column had been pre-treated with sulfuric acid.

The absorption curves for the yellow pigment from the blackened tubers (Fig. 2, A and B) give indication of two maxima at approximately 3,350 Å and 2,650 Å. The position of the first maximum is somewhat better defined in the curve for the pigment obtained from columns which had been treated with sulfuric acid prior to use (Fig. 2, A) than in the curve for the non-treated column (Fig. 2, B). The maximum in the far ultra-violet, however, is more pronounced and also shows greater intensity under the latter conditions (Fig. 2, B).

The absorption curve for the yellow pigment from non-blackened tubers (Fig. 2, C) is similar in general aspect to those of the other yellow pigments. In order to avoid confusion between the experimental points obtained for the absorption of the three preparations of yellow pigments, the absorption curve for the pigment from non-blackened tubers (Fig. 2, C) was displaced somewhat by using a different set of ordinates. There is an apparent maximum in this absorption curve at approximately 3,400 Å and a fairly sharp one at 2,650Å. The band nearer the visible region is relatively less intense but otherwise not significantly different from the corresponding band in the other spectra. Of the three curves the two that are most alike are those for the yellow pigments obtained from non-treated columns (Fig. 2, B and C).

The relationship between all the spectra of the pigments is shown best when the experimental points are not given and the curves are not displaced (Fig. 3). The absorption bands for the gray pigment appear to be broader than those for the yellow pigments; otherwise, the absorption curves are not very different. The presence of a plateau instead of a real maximum in the far ultra-violet may be due to experimental conditions rather than any real difference since such a plateau appears also in one curve for the yellow pigment (Fig. 3, A). As explained elsewhere (p. 229) the position of the curves with respect to the logarithm of the extinction coefficient has no significance in the present work.

The absorption spectra for both the gray and yellow pigments show the two bands reported by Skarżyński (1939) as characteristic of a flavone-like substance. To which flavone derivative they are related is not evident from these data. It was necessary to use aqueous sodium hydroxide solutions in the present investigation, whereas Skarżyński (1939) identifies the various flavone compounds by the spectra taken in alcoholic solutions. Such data as have been presented by Lajos and Gerendas (1937) for sodium hydroxide solutions indicate that while there is a greater separation of the bands in that solvent than in alcohol, the two characteristic bands do persist in dilute alkaline solutions.

SUMMARY

Some properties, including the ultra-violet absorption spectra, of melanin and of the pigments from blackened and non-blackened potatoes have been compared. A gray pigment and a yellow, flavone-like pigment have been separated from water extracts from discolored potatoes by chromatographic adsorption. A similar yellow pigment was obtained from non-blackened tubers. The properties of the gray pigment and the blackened portions from which it was separated were unlike those of the melanin from raw potatoes. The gray pigment was quite unstable and appeared to be related to the yellow flavone derivative.

ACKNOWLEDGMENT

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EXPERIMENTS WITH ANTIOXIDANTS FOR PREVENTING FLAVOR DETERIORATION IN CANNED ORANGE JUICE[1]

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In a previous publication Nolte and von Loesecke (1940) showed that the petroleum ether-soluble material in aged, canned Valencia orange juice had undergone oxidative changes with the creation of hydroxy acids and other decomposition products from the fats and resins, and that the former had become rancid. In view of the evidence presented, it was believed that off-flavors developing in canned Valencia orange juice were due, at least to some extent, to these oxidative changes. The present paper deals with an attempt to prevent these oxidative changes, and hence to prolong the "life" of the canned product, by means of antioxidants. In this connection it may be mentioned that orange juice itself contains natural antioxidants. The most important of these are probably sulfhydryls, ascorbic acid, and sterols. It is quite likely, too, that the sugars, certain amino acids, and proteins have antioxygenic properties, Lea (1939). On the other hand, the juice contains pro-oxidants in the form of traces of copper, iron, and zinc.

The antioxidants used were those believed to be the most promising in the light of recent researches by others. However, such researches have shown that the antioxygenic value of a substance, although high in a particular system, may disappear when this substance is employed in a slightly different system. This seems to be particularly true in systems containing water, as would be the case with orange juice. In the selection of antioxidants their toxicity was considered, but it should be pointed out that some of the antioxidants used in these experiments have not been proved harmless when consumed with foodstuffs. Should such a material be used in foods offered for public consumption, it will be the responsibility of each manufacturer who proposes to use it to establish the harmlessness of the substance before doing so. This is necessary in view of the Federal Food, Drug, and Cosmetic Act.

The kinds and quantities of antioxidants used are indicated (Table 1); these quantities were calculated as being more than sufficient to combine with the oxygen in the juice. In some instances, of course, this could not be calculated. With the exception of "Methyl Glucamine,"[2] which was recrystallized three times from water, none of the antioxidants was purified before use. The different chemicals were weighed into No. 4 gelatin

[1] Agricultural Chemical Research Division Contribution No. 39.

[2] Supplied by E. I. du Pont de Nemours.

capsules which were subsequently placed in the cans. None of the antioxidants in the proportions used caused a foreign taste in the juice. As controls, empty capsules were used. The gelatin was found to be inert.

EXPERIMENTAL PROCEDURE

To duplicate the newer commercial practices, the packs were made in a canning plant as part of a regular run. Briefly, the process used consisted of automatically extracting the juice, screening, deaerating, flash-pasteurizing at 87.8°C.(190°F.), filling at about 85°C.(185°F.) into both enameled and plain tin cans, and sealing in either nitrogen or air. The oxygen content per "211 x 414" can varied from .03 to .09 c.c. The juice had a Brix hydrometer reading of 10.9°, acidity of 1.35 per cent as anhydrous citric acid, pH of 3.7, and an oil content of .098 per cent by

TABLE 1

Antioxidants Used Experimentally to Prevent Flavor Deterioration in Canned Valencia Orange Juice

Antioxidant	Quantity used—parts per million parts of canned juice
Asparagine	2, 4 and 6
α Naphthol	2, 4 and 6
Chloresterol	15, 22 and 30
Methyl-p-aminophenol (Elon)	2, 4 and 6
d-Isoascorbic acid	30, 60 and 100
Hypophosphorous acid	2, 4 and 6
Lecithin	2, 4 and 6
"Methyl Glucamine"	200 and 500
Resorcinol	2, 4 and 6

volume. The oil content was unusually high and significantly impaired the quality of the juice. Practically all packs of Florida citrus juices after the January, 1940, freeze showed high oil content. The canned juice was stored at room temperature which varied during the season from —4.5 to 35.5°C.(24 to 96°F.).

Methods of Analysis: Each month the product was subjected to the following tests: sulfhydryl, Täufel's ketone reaction, and peroxide value of the petroleum ether-soluble material of the juice. Since this last test called for modification of the standard technique, it will be described in some detail. The juice from a "211 x 414" can (which was sufficient for the examination) was fed slowly through a supercentrifuge. It was followed by twice its volume of water. The sludge was removed from the bowl and mixed with sufficient acetone to form a pasty mass. This was transferred to a separatory funnel and extracted five times with 30-c.c. portions of petroleum ether. The petroleum ether was washed three times with water and the washed ether was filtered into a weighed 250-c.c. Erlenmeyer flask. Excess ether was removed by distillation and the residue dried at 100°C.(212°F.) for one hour under a blanket of carbon dioxide. The residue thus obtained amounted to from .04 to .1 gram, depending upon the care used in centrifuging and extracting. The weighed

residue was dissolved in 10 c.c. of solvent (six parts glacial acetic acid, four parts chloroform); one drop of 10 per cent ammonium molybdate solution was added as a catalyst and was followed by approximately .1 gram of solid potassium iodide. The flask was placed in a boiling-water

TABLE 2

Protective Factors of Antioxidants as Applied to Canned Florida Valencia Orange Juice

Antioxidant	Protective factor (PF)	
	In enameled cans	In plain tin cans
d-Isoascorbic acid		
30 p.p.m.	2.0	2.0
60 p.p.m.	2.0	2.0
100 p.p.m.	2.0	2.0
Asparagine		
2 p.p.m.	2.0	2.0
4 p.p.m.	2.0	2.0
6 p.p.m.	2.0	2.5
Lecithin		
2 p.p.m.	1.0	1.0
4 p.p.m.	2.5	2.0
6 p.p.m.	2.5	2.0
Resorcinol		
2 p.p.m.	1.5	1.0
4 p.p.m.	2.0	2.0
6 p.p.m.	2.0	2.0
Hypophosphorous acid		
2 p.p.m.	1.0	1.5
4 p.p.m.	1.0	2.0
6 p.p.m.	2.5	2.0
Chloresterol		
15 p.p.m.	1.0	1.5
22 p.p.m.	1.0	1.5
30 p.p.m.	2.5	1.5
"Methyl Glucamine"		
200 p.p.m.	1.0	1.5
500 p.p.m.	1.0	2.0
α Naphthol		
2 p.p.m.	3.5	2.5
4 p.p.m.	3.5	2.5
6 p.p.m.	3.5	2.5
Methyl-p-aminophenol (Elon)		
2 p.p.m.	1.0	1.5
4 p.p.m.	1.0	2.0
6 p.p.m.	2.0	2.0

bath for exactly one minute from the time potassium iodide was added. After cooling, the mixture was titrated with .002N sodium thiosulfate, using a microburette. The peroxide value is expressed as the number of cubic centimeters of .002N sodium thiosulfate required per gram of extract.

DISCUSSION OF RESULTS

Initially, all of the samples gave a strongly positive sulfhydryl test comparable to that of fresh juice. After a storage period of one month

the test was weaker. In two months it became negative, but after reducing the canned product with potassium cyanide, it again became positive. Since sulfhydryls are themselves considered antioxidants—Josephson and Doan (1939)—their destruction must result in a loss of at least a part of any natural antioxidant properties the juice might have. None of the added antioxidants in the amounts used either retarded or prevented the destruction of sulfhydryls naturally present in the juice.

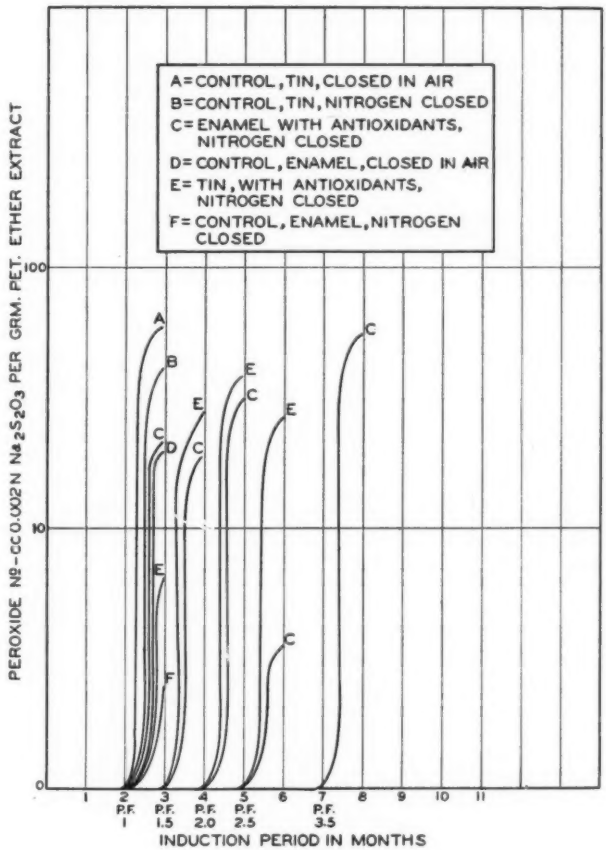

FIG. 1

The results of Täufel's higher-ketone test coincided quite generally with those of the sulfhydryl test; i.e., they were negative for the first month of storage and positive after the second month. As pointed out in a previous paper by Nolte and von Loesecke (1940), the formation of higher ketones may, in some instances, be detected by taste and may account for part of the off-flavors in aged, canned orange juice. It was

believed that these ketones resulted from the oxidation of higher fatty acids, as well as aldehydes, in the essential oils of the juice. For instance, citral, which may be present in Florida Valencia orange juice—Nelson and Mottern (1934)—through the incorporation of peel oil, will yield 2 methyl $\triangle^2$ heptanone and carbon dioxide when oxidized.

Since there was a difference in the oxygen content of the different cans, owing to the commercial technique employed, peroxide values of duplicate or triplicate cans from the same lot varied by as much as 15 per cent.

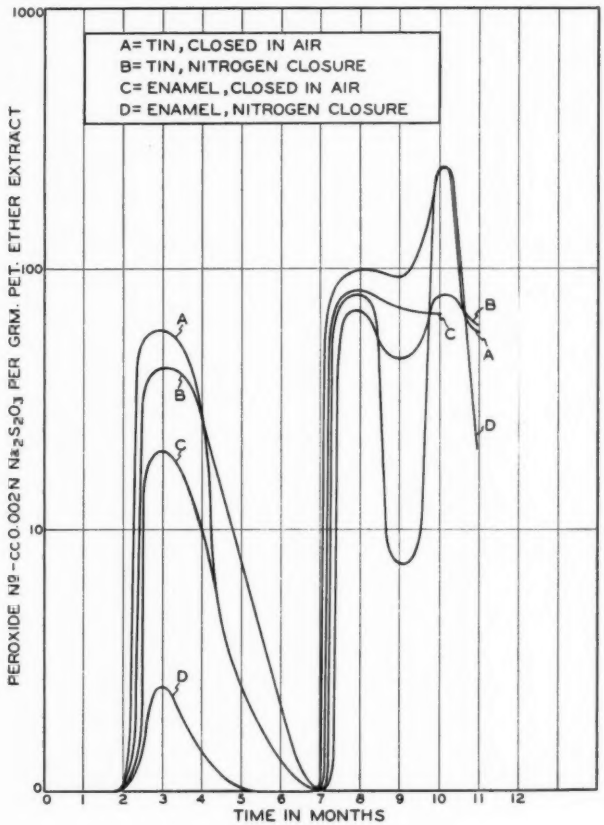

FIG. 2

It was found that the antioxidants could be tabulated according to their "protective factor" (PF). This term is defined as the ratio of the length of the induction period (in months) of the juice containing antioxidant to that of juice without antioxidant (Table 2). An antioxidant having a protective factor of 1.0 would be considered inactive.

Since several of the antioxidants used have similar protective factors (Table 2), it was deemed justifiable to combine the results obtained with

such antioxidants and plot them as one curve. This is the procedure followed and represented (Fig. 1); it will be observed that the peroxide numbers of the lots vary, and this is quite likely due to the fact that in some cans there was three times as much oxygen originally present as in other cans. Nevertheless, the curves indicate that the induction periods are prolonged from one to five months when using certain antioxidants.

Data for lots containing antioxidants are similar to those presented in Fig. 2; but, to conserve space, they are not plotted. The essential differ-

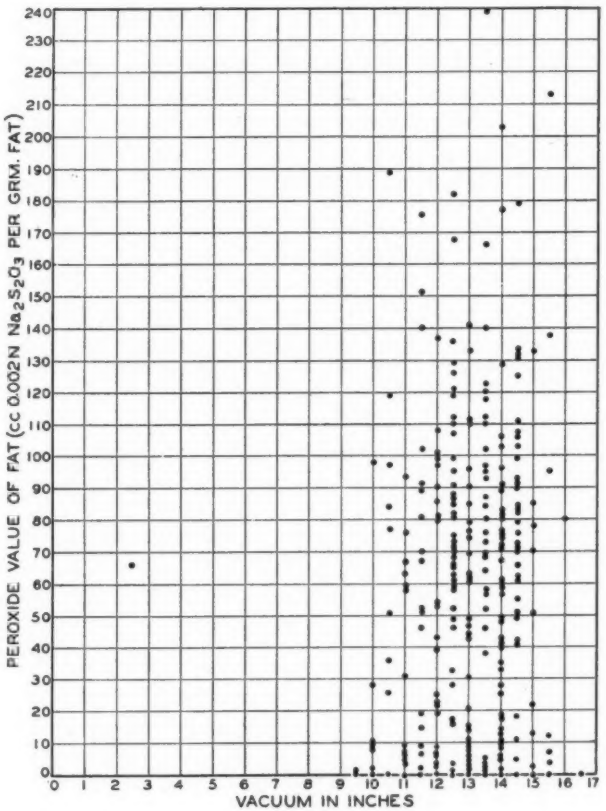

FIG. 3

ence between the curves of the control lots (Fig. 2) and those containing antioxidants is the delay in the first induction period of the latter as indicated in Fig. 1. It is evident that the curves of the control lots reveal three induction periods. The first two are distinct, but the induction period after nine months is less apparent, owing to the fact that it occurs before peroxide decomposition of the second induction period has been completed. These three induction periods may be explained by the presence in the

orange juice used of three substances—terpenes, fats, and resins—which are all capable of forming peroxides. Terpenes and fats form unstable peroxides. In a limited supply of oxygen, after the induction period is passed, the ability of terpenes and fats to form peroxides gradually diminishes as the free oxygen is consumed; decomposition of peroxides already formed then takes place and finally the peroxides disappear. The peroxides of resins, on the other hand, are known to be relatively stable. Since terpenes have been used as antioxidants for fats—Nakamura (1933)—it may be assumed that the terpenes present in the orange juice oxidized first, followed by the fats and resins. The order of oxidation, however, is probably of little importance.

While none of the antioxidants used in this study entirely prevented oxidation and flavor deterioration in the experimental packs, the results indicate that the induction period could be prolonged by certain antioxidants. It should be pointed out, however, that no correlation could be found between peroxide value of the petroleum ether extract of the juice and taste of the juice. Such a correlation was made difficult by the adventitious introduction of peel oil into the experimental packs. Further work is being carried on with the use of other materials having antioxidant properties.

Juice packed in enameled cans possessed a better flavor than that packed in plain tin cans, and this difference was first apparent in three months. It is doubtful, however, whether this difference would be detected by the average consumer. The justification for increased packing cost in enameled cans may, therefore, be questioned.

Nitrogen closure of the containers did not seem to improve the keeping quality of the juice under the conditions of the experiment. In general, however, the peroxide value of the petroleum ether-soluble material from orange juice packed in cans which were nitrogen closed was less than that from juice packed in cans closed in air.

There was no correlation between the vacuum in the cans and the peroxide number of the petroleum ether extract of the juice (Fig. 3).

SUMMARY

Nine antioxidants in different concentrations were added to Florida Valencia orange juice in an attempt to prevent off-flavor development in the canned product. Although some of the antioxidants prolonged the induction period as measured by the peroxide value of the petroleum ether-soluble matter of the canned juice, no correlation could be found between the peroxide value and taste of the juice. Such correlation was made difficult by the adventitious introduction of peel oil into the experimental packs. As measured by the peroxide number of the petroleum ether-soluble material of canned orange juice, there were three induction periods. This is believed to be due to the successive oxidation of terpenes, fats, and resins in the canned juice, probably in the order named.

None of the antioxidants in the amounts used prevented the destruction of sulfhydryl compounds or the formation of higher ketones in the juice.

Juice packed in enamel cans had a better taste than that packed in plain tin cans, but it is questionable whether the difference could be detected by the average consumer.

There was no correlation between vacuum in the cans and peroxide value of the petroleum ether extract of the canned juice.

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MULTIPLICATION OF BACTERIA IN WATER AND ITS SIGNIFICANCE IN FOOD SPOILAGE

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It has long been known that bacteria tend to multiply in ordinary water which has been left standing at room temperature. In a book on microörganisms in water, Frankland and Frankland (1894) devoted a whole chapter to the multiplication of bacteria in water. In this volume are recounted the experiments of Frankland himself and others, showing that in water from deep wells, springs, rivers, lakes, and even distilled water the bacterial increase is surprisingly large. In more recent years similar observations have been made by other workers—Albert, Hinman, and Jordan (1917); Eijken (1918), and Tanner and Schneider (1935)—but in almost every instance the problem has been considered only from the standpoint of public health. As a result of these studies, many species of microörganisms have been classed as "harmless water bacteria" and have been given little consideration.

Water is an essential in almost all food industries and if the bacterial flora in water can increase from dozens to millions by simply remaining at ordinary temperatures for a day or so, this may have a very great significance quite apart from the public-health standpoint. It has been the object of the work recorded in this paper to re-examine this question in terms of food spoilage and to determine not only the numbers, but more particularly the types, of bacteria that are able to multiply in ordinary tap and well waters.

Much of the experimental work has been carried out with the tap water at the Ontario Agricultural College. As this water is untreated and comes from an ordinary drilled well, it is considered fairly typical of the water supplies of many rural creameries and other food-manufacturing establishments. Water supplies have also been examined from dairies, creameries, and farm wells from many parts of Ontario.

EXPERIMENTAL PROCEDURE

In general, the procedure has consisted of obtaining water samples at the source of their supply, incubating them at room or at fixed temperatures, and making periodical plate counts to determine the numerical changes in their microflora. From the colonies developing on the plates, studies were made to determine the predominant types. It was found that many of the organisms in water grow relatively slowly on standard beef agar, and for this reason plates incubated at 37°C.(98.6°F.), 25°C.(77°F.), or at room temperature were never counted until after four days of incubation; those incubated in an ice box at 2 to 5°C.(35.6 to 41°F.) were left at least seven days. The containers for holding the water consisted mainly of chemically

clean, sterilized, screw-cap bottles. Some tests, however, were made with stainless-steel vats, glass-lined tanks, milk cans, and crocks and with various sized containers holding from 25 milliliters to 360 liters.

Characteristics of the College Water: The College water supply was obtained from a drilled well 220 feet deep, which pumps about 170,000 gallons a day. The water is not chlorinated, nor treated in any other manner. Chemical analysis of an average sample as it comes from the tap shows it contains the following: (NO_3^-)—none; (NO_2^-)—none; (NH_4^+)—trace; (Cl^-)—trace; (Fe^{+++})—none; (Fe^{++})—none; (Ca^{++})—60 p.p.m.; (Mg^{++})—250 p.p.m.; soluble substances—volatile .01 per cent, nonvolatile .03 per cent. From a 100-ml. sample there is a very slight trace of charring. The

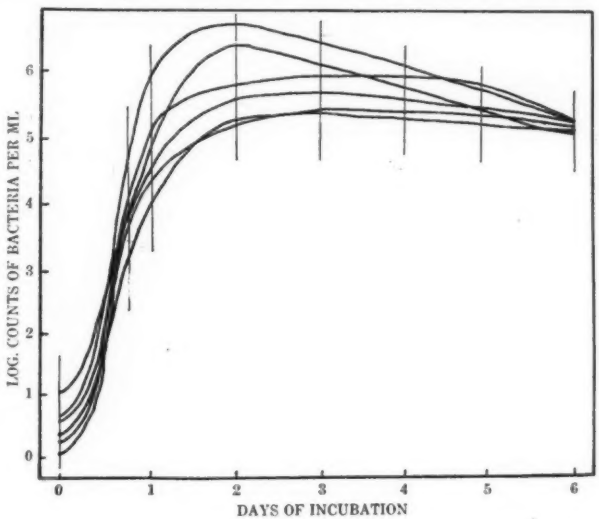

FIG. 1. Growth curves showing the numbers of bacteria developing in six samples of College tap water when held at room temperature, 25°C.(77°F.), for a period of six days. Counts were made from the water at 0, 18, and 24 hours and daily thereafter.

average bacterial count of the water, when taken at the pump head, was less than one per milliliter, though some bacteria were invariably present in larger samples. Taken from the taps in the College buildings, the counts varied from less than one to about 50 per milliliter; the average of 40 counts gave 10 per milliliter.

Numerical Increase at Room Temperature: Six 150-ml. samples of tap water were taken on different days and incubated at 25°C. The increases in bacteria over a period of six days, as indicated by plate counts on standard beef-extract agar, incubated at 25°C. are shown (Fig. 1). The average initial count of these samples was four per milliliter; at 48 hours the average count was 1,972,000; at six days it had dropped to 223,000. After 40 days the average count of four of these six samples remained at about 300,000 per milliliter; at 60 days it had dropped to 40,900. Typical curves, similar to those shown in Fig. 1, were obtained for a great many other samples of

tap water held in containers at 25°C. The maximum counts were usually obtained between two and five days; they never exceeded 10 million and were rarely under 100,000 per milliliter. The biggest variations were observed in the counts showing the rate of decline in the growth curves. It is considered that the species of bacteria present in the water may have had something to do with this difference.

The growth curves are shown (Fig. 2) of the bacteria in four samples of tap water; but instead of being held at 25°C., they were left in the laboratory where the temperature ranged between 21 and 31°C.(69.8 and 87.8°F.). To compare these with the development of bacteria in water from other sources, counts were made on 12 samples of water procured from

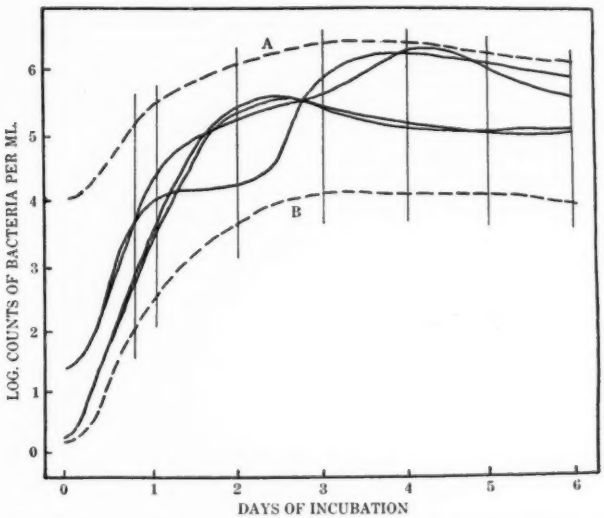

FIG. 2. Growth curves showing the numbers of bacteria developing in four samples of College tap water when left in a room where the temperature varied between 21 and 31°C.(69.8 and 87.8°F.). The area enclosed between the lines A and B contains the growth curves of water samples from 12 creameries incubated under similar conditions.

dairies within a 30-mile radius of Guelph. These were also held at the varying temperature of the room. The band included by the lines A and B (Fig. 2) contains all the growth curves of the bacteria in the dairy-water samples. These indicate that the growth rates of bacteria tend to be similar in water from different sources.

Effect of Temperature on Growth of Bacteria in Water: Under normal conditions the water in a dairy is often subjected to a wide range of temperatures. The effect of such changes on the growth of bacteria in the water was studied by the following methods: (1) the water was held at different temperatures and the plates from which the counts were made were incubated at corresponding temperatures; (2) the water was held at one fixed temperature and the plates were incubated over a range of temperatures;

(3) the water was held at various temperatures and the plates were all incubated at 25°C.

To a considerable extent the results of these temperature experiments varied with the characteristics of the organisms initially present in the water. However, the general results from a large number of tests with normal, low-count water may be summarized as follows:

(a) When the water itself was held between 2 and 5°C. (35.6 and 41°F.), the results were the most unpredictable and the growth rates were subject to the greatest variation. Usually the development was extremely slow. Occasionally, however, after a period of almost dormancy, the counts would suddenly rise from less than a hundred to several hundred thousand. The

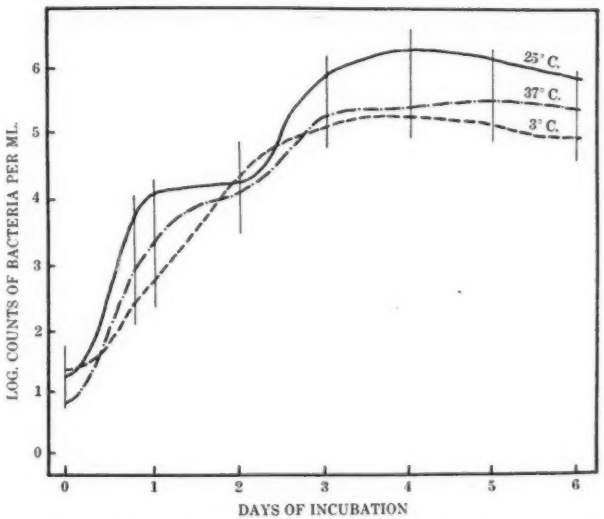

FIG. 3. Numbers of bacteria developing in a sample of College tap water, as indicated by plate counts, where the plates were incubated at 3, 25, and 37°C. (37.4, 77, and 98.6°F.). The water itself was kept at the varying temperatures of the laboratory which ranged between 21 and 31°C. (69.8 and 87.8°F.). The counts were made from the water at 0, 18, and 24 hours and daily thereafter.

plate counts from water held at this temperature always gave higher counts when incubated at 25°C. (77°F.) than at 3°C. (37.4°F.).

(b) In most instances where the water was held at 25°C., the organisms which developed were capable of growing on the beef-agar medium over a remarkably wide range of temperatures, 3 to 37°C. (37.4 to 98.6°F.). Typical growth curves are shown (Fig. 3) of bacteria in a normal sample of College tap water held at 25°C., as indicated by plate counts made at 3, 25, and 37°C.

(c) Where the water was held at 37°C., the bacterial development was somewhat similar to that when it was held at 25°C. Comparative tests showed generally, however, that the initial rise was slower, the maximum count was lower, and the subsequent decline was more rapid.

From these tests the one thing that stands out as important is the fact that the microflora which develops in water held at room temperature has a temperature range almost to the freezing point. This confirms an observation made previously by Castell and Garrard (1941) that 87 out of 100 samples of rural water sent in for analysis contained large numbers of bacteria which grew readily on plates incubated at 3°C.

Growth in Distilled Water: Two 150-ml. samples of water, obtained from a copper still, were each inoculated with one loopful (.001 ml.) of incubated tap water. For several days the organisms remained dormant and then the counts rose slowly to maxima of 144,000 and 108,000 per milliliter at 16 days. After 53 days the average was 23,100 per milliliter.

Size of Containers: It may be objected that most experiments on the growth of bacteria in water are carried out with small, closed containers and that the results do not apply to the large vats used by dairies and other

TABLE 1

Multiplication of Bacteria in Tap Water Stored in Different Sized Containers and Held at Room Temperature, 21 to 31°C.(69.8 to 87.8°F.)

Age of sample	Amount of water						
	25 ml.[1]	150 ml.[1]	450 ml.[1]	20 liters[1]	176 liters	243 liters[2]	
						Top	Bottom
Initial	5[3]	3	14	2	70	88	78
24 hours	34,000	13,500	40,000	6,000	37,000	23,000	660
48 hours	1,900,000	780,000	570,000	39,000	170,000	250,000	925,000
72 hours	860,000	960,000	620,000	80,500	680,000	480,000	3,120,000
96 hours	660,000	1,080,000	2,200,000	150,000	960,000	720,000	2,900,000
7 days	260,000	300,000	146,000	Omitted	2,000(?)	81,000	900,000

[1] Portions of one well-mixed sample. The remaining containers had water from the same source drawn immediately afterwards. [2] The water in all containers, except this 243-liter vat, was well mixed before samples were taken. [3] Bacterial plate count per milliliter of water.

food manufacturers to store their water supplies. Such reasoning is confirmed by the work of Zobell and Anderson (1936) with bacteria in sea water. They found appreciably more bacteria per milliliter developing in small containers than in large ones. In order to throw more light on this point, six containers of varying sizes were used: 25, 150, and 450 milliliters; 20, 176, and 342 liters.

In each instance the containers were filled with the College tap water, and those from 25 milliliters to 20 liters were filled from one well-mixed sample. The 176-liter vat had an automatic mixer, and both the large vats were well covered to prevent contamination from the air. The samples were all incubated at room temperature and the plates were incubated at 25°C. Except in the case of the largest vat, samples were taken only after the contents had been thoroughly mixed so as to obtain representative samples. With the largest vat, samples were taken both from the top four inches and from the bottom four inches of the vat. The results (Table 1) indicate that where the initial number of bacteria was approximately the same (i.e., the 25-milliliter to 20-liter lots) the size of container may have had some influence on the rate of growth. On the other hand, they also indicate that in

spite of the size of the containers, all the samples show very large numbers of bacteria after standing for two or more days.

Flowing Water: Under practical conditions, water in storage vats rarely lies dormant; it is constantly being replaced, and the rate of replacement varies considerably. Experiments were conducted to find out the effect of this constant replacement on the multiplication of bacteria in the water.

A 20-liter container was arranged so that a constant supply of tap water was delivered about six inches below the surface and the outflow siphoned off the surface. Below the intake was a column of water 15 inches deep. Except for the bacteria entering in the tap water all outside contamination was prevented. Counts were made on the water siphoned off the surface as well as from the water at the bottom of the container. In different experiments the rate of flow varied from three to 40 liters per hour. The counts showed that at the bottom of the container the bacteria multiplied

TABLE 2

Inhibiting Action of Sodium Hypochlorite on Growth of Bacteria in Tap Water Measured as Available Chlorine at Start of Experiment

Age of samples	Amount of available chlorine (p.p.m.)					
	0	.12	.25	.37	1.3	2.5
Initial	21[1]	32	40	36	33	45
24 hours	6,400	8	0	41	0	0
48 hours	41,000	146	7	207	0	0
72 hours	182,000	40,000	23,000	171,000	0	0
96 hours	38,000	150,000	180,000	210,000	0	0
120 hours	80,000	550,000	1,390,000	800,000	0	0

[1] Bacterial plate count per milliliter of water.

much as in any other stored sample; at the surface of the water, although greatly in excess of the numbers in the tap water itself, the counts never reached over 3,000 per milliliter. As would be expected, the faster the flow the lower the count. This suggests that in storage tanks, if the inlet and outlet are not properly placed or if the rate of replacement is slow, bacteria may readily develop in the lower portions of the tank.

Inhibiting Action of Chlorine and Sodium Chloride: The problem of *inhibiting the growth* of a relatively small number of bacteria may be quite different from the problem of disinfecting water already contaminated from some outside source. Preliminary experiments were carried out with sodium hypochlorite in College tap water. As shown (Table 2), from .12 to .37 p.p.m. of available chlorine did not altogether prevent the organisms from growing. It did, however, retard growth for 48 hours; but this was followed by rapid increases which, in all instances, greatly outnumbered the maximum counts of the untreated samples. Over 1.3 p.p.m. of chlorine inhibited all growth up to 21 days, when the tests were terminated.

Sodium chloride had a somewhat similar action. Three per cent (wt./vol.) salt added to water inhibited growth in all the tests made with College tap water. Two per cent, or less, retarded growth for various lengths of time from several hours up to several days; but invariably when multi-

plication began, the maximum numbers were greater than those in the untreated samples.

This portion of the work, dealing with the inhibiting action of various substances on the *growth of bacteria in water,* is being continued and will be reported more fully in a subsequent paper.

Characteristics of Bacteria That Multiply in Water: The types of bacteria that *multiply* in water and types that are *found* in water under natural conditions are not, necessarily, the same. The latter may be contaminants from soil, air, or other sources. For this reason all attempts to study the characteristics of bacteria were made from water samples where the initial number of cells were few but had increased many thousandfold by actual growth in the water. Two different methods were used in this survey of culture types. Incubated water samples were plated on differential media, giving a rough estimation of the percentage of lipolytic, proteolytic, "oxidase"-producing,[1] and fluorescent colonies in a large number of samples. Plates were also incubated at 3°C.(37.4°F.) to give some idea of the percentage of psychrophilic types, and many colonies were examined for the Gram reaction. In addition to these more general observations 60 pure cultures were isolated for more detailed study.

Gram-positive organisms were never found among those actively multiplying in water, although they were frequently found in the water to begin with. In almost every instance, the longer the water was held the more limited were the number of types that developed on the plates. After a week or more of incubation almost 100 per cent were strongly "oxidase"-positive, offensive-smelling colonies resembling members of the *Pseudomonas* genus. Some had the characteristic green pigment of the fluorescent group; others were shades of pink, brown, orange, yellow, cream, or white or were colorless.

Those developing in numerous samples of the College tap water after 24 hours' incubation showed at least 85 per cent "oxidase"-positive, 85 per cent proteolytic, and 75 per cent strongly lipolytic. Apparently they were restricted to two or three species. Examination of bacteria developing in water samples from 125 dairy-water supplies and several hundred farm wells gave a varying, but somewhat similar, picture. "Oxidase"-positive, proteolytic, offensive-smelling colonies always predominated. Notable exceptions were the majority of dairy-water samples where the water had been previously chlorinated; some of these, however, contained many organisms of this type. It was also observed that a very large percentage of the organisms growing in these water samples developed colonies on standard beef-agar plates incubated at ice-box temperatures of 2 to 5°C.(35.6 to 41°F.).

In selecting the 60 cultures for further study the object was to obtain as many different types as possible. It should be borne in mind, however, that certain of these types were much more abundant than others, not only in individual samples but in the samples as a whole. A summary of the characteristics of the 60 cultures (Table 3) indicates that the most important ones are the general predominance of gram-negative, alkaline-forming, "oxidase"-positive, proteolytic and lipolytic organisms with a relatively

[1] For details of this test see the senior author's article in *Food Research* **5**, 215-222 (1940) or *The Canadian Dairy and Ice Cream Journal* **19**, 26-28 (1940).

low-temperature range, and the almost entire absence of organisms capable of fermenting carbohydrates and gram-positive cocci or spore-forming bacilli. As indicated by the growth on Koser's synthetic, sodium-citrate medium, the majority of these organisms are able to utilize salts of organic acids as sole sources of carbon. Seventy-five per cent of the 60 cultures were small to medium-sized rods; the remainder were coccoid types or cells showing considerable pleomorphism.

Inoculation of Sterile Tap Water With Identified Species: Sterile tap water was inoculated with aqueous dilutions of pure cultures. These were

TABLE 3

Summary of Characteristics of 60 Cultures of Bacteria Found Growing in Water Samples at 25°C.(77°F.)

Reaction	Positive cultures
	pct.
Gram reaction	0.0
Production of indol	3.3
Acid and gas from dextrose[1]	6.6
Acid and gas from sucrose	6.6
Acid and gas from lactose	6.6
Hydrolysis of starch	10.1
Fluorescent colonies	21.3
Visible proteolysis in plain milk	30.0
Proteolysis—gelatin agar plates	41.3
Proteolysis—skimmilk agar plates	50.0
Nitrites from nitrates	45.0
Motility	66.6
Reduction of resazurine in beef broth at 3°C.(37.4°F.)	71.6
Alkaline reaction in Koser's citrate agar medium	73.3
Hydrolysis of butter fat	76.6
"Oxidase" reaction	78.3
Hydrolysis of tributyrin	100.0

[1] Of the remaining 93.4 per cent of the cultures only a few were able to produce traces of acid from these sugars. None of these gave a typical acid reaction with B.C.P. as an indicator.

incubated at 25°C.(77°F.) and plated out periodically. Where multiplication appeared to be taking place, suitable tests were made to ensure that the results were not due to some accidental contamination. The initial cell content of these samples, as indicated by the plate counts, varied between 10 and 5,000 per milliliter.

The following species showed active multiplication in the water, reaching counts of over 500,000 per milliliter: *Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas fragi, Aerobacter aerogenes, Serratia marcescens,* and *Achromobacter lipolyticum. Pseudomonas putrefaciens* gave variable results, showing in one instance counts of from 200 to 73,000 per milliliter after 160 hours, but it apparently grew better in water when accompanied by certain other organisms.

Escherichia coli, Proteus vulgaris, Alcaligenes viscosus, Staphylococcus aureus, Staphylococcus citreus, Sarcina lutea, Micrococcus conglomeratus, Bacillus subtilis, Bacillus mycoides, Bacillus graveolens, Bacillus panis, and

Bacillus mesentericus showed no significant increases over a period of 20 days.

When one considers the difference in the nutritive value of the media on which these organisms are usually grown, compared with that of tap water, it is surprising that any growth at all takes place in the water and more especially in distilled water. It seems probable, however, that by gradually reducing the nutrient value of media, many organisms which would not grow on immediate transfer may become accustomed to the scanty fare of tap water. In fact, just such an experiment was performed by Frankland and Ward (1892) with the typhoid organism, and they found that by "gradual training in more and more aqueous culture-media" the organisms did exhibit "distinctly more vitality in potable water than bacilli which are at once transferred into water from highly nutritive solid media like agar or peptone jelly." They were not, however, able to produce a typhoid culture which would flourish in river water free from organic matter.

These considerations, together with observations which appear to indicate the associative action of certain bacteria in water, have led the authors to lay more stress on the bacteria found growing in water under natural conditions than on the results of these inoculations with stock cultures.

SUMMARY AND DISCUSSION

The data presented in this paper show that water from several hundred deep and shallow wells, having an initially low bacterial content, contained organisms which underwent extensive multiplication when the water was enclosed in a container. The type of container and its size (up to 80 gallons) appeared to have relatively little effect on the numbers of bacteria which developed. Why these same organisms do not develop in the well itself is an interesting problem. A similar question is presented by the subsequent increase of bacteria when sea water is transferred to a container.

The types of bacteria that develop have been observed to include mainly non-saccharolytic, gram-negative organisms having the general characteristics of the genera *Alcaligenes, Pseudomonas*, and *Achromobacter*. A large proportion of these are lipolytic, proteolytic, and "oxidase"-positive. Many are active at temperatures not far above freezing. That such organisms are injurious to stored meats, fish, butter, and other non-acid, protein, or fatty foods seems self-evident.

It was interesting to note that initiation of the growth of these organisms at low temperatures was very often governed by the number of cells present.

As early as 1894, Frankland and Frankland (1894) spoke of "the inadmissibility of delaying the bacteriological examination of a sample of water beyond a few hours, even if the sample be packed with ice." This observation has been confirmed by many workers since then, and the present paper suggests that this is particularly true where the analysis is to be considered in terms of food spoilage rather than for potability of the water. During these investigations water samples were analyzed directly as they came from the well at many dairies and again when similar samples were sent into the laboratory through the mail. The results were so divergent as to make analyses of mailed samples appear ridiculous. A similar error has been to

judge a water supply by one small sample taken at one place in a water-supply system. The number of organisms not only varies from place to place (i.e., top or bottom of a vat, pipes, etc.) but also varies from time to time, depending upon the temperature, rate of flow, and other factors. Observations were made in which the bacteria in dead-end or unused pipes increased from very low counts to many thousands per milliliter.

Preliminary experiments have indicated that initial concentrations of one or more parts per million of chlorine markedly reduced the number of organisms and there was no evidence of aftergrowth after a period of 120 hours. Lower initial concentrations of chlorine (.12 to .37 p.p.m.) effected a temporary decrease or lag in growth followed by a rate of increase which was greater than that in the control (unchlorinated) water.

Inoculations of sterile tap water with pure cultures of known organisms appeared to confirm the observation that growth in water of low organic content was restricted to certain groups of gram-negative bacteria. Known species of *Pseudomonas* were particularly active in water.

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EFFECT OF FAST-FREEZING UPON BACTERIAL FLORA OF MACKEREL

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Much work has been done upon the effect of fast-freezing on the microbial flora of various products but the greater number of these experiments have dealt with vegetable materials. This preponderance of interest in plant products is due to the facts that more of them are processed by this method than are meat and fish and that some fruits and vegetables contain a higher microbial content than do meat, fish, or poultry. The research which is to be detailed here concerns mackerel when fast-frozen.

WORK OF PREVIOUS INVESTIGATORS

Smart and Brunstetter (1937) and Smart (1937a.b), (1939a,b) reported on the effects of fast-freezing upon peas, beans, sweet corn, spinach, kale, and blueberries and made the following statements: Storage of blueberries over a period of nine months at —17.8°C.(0°F.) resulted in a decrease of 59.7 per cent in microbic count, but over a similar period of time at a temperature of —6.7°C.(20°F.) this reduction amounted to 99.9 per cent. Seven months holding at —9.4°C.(15°F.) destroyed approximately 99 per cent of the organisms. The same author, while working with other vegetables, stated that storage for five to seven months at —17.8°C. was attended by a reduction of 98.1 per cent of microorganisms in green and wax beans, 85.6 per cent in lima beans, and 57.3 per cent in sweet corn. Spinach and kale, frozen and stored at —9.4°C., showed a decrease of microbial count of the order of 80 per cent at the end of seven months and approximately 93 per cent at the end of 15 months. From figures quoted by Lochhead and Jones (1936) we calculated decreases of 91.1 per cent in dry-pack peas, 93.4 per cent in dry-pack beans, and 98.1 per cent in brine-pack corn in six weeks at —17.8°C., although a portion of the decrease in count of the peas and beans was due to blanching. Lochhead and Jones (1938) reported species of Micrococcus and Flavobacterium as the most numerous survivors. In a study of the freezing redistribution of microorganisms MacFarlane (1940) showed that there was a greater decrease in viable forms in cider at —20°C.(—4°F.) than at —10°C.(14°F.) but that the reverse was true for sirup-packed raspberries. Prescott and Tanner (1938) reported the survival of small numbers of Salmonella and *Clostridium botulinum* at —20 to —40°C.(—4 to —40°F.) for a few weeks when large doses were inoculated to spinach.

Birdseye (1929) quoted figures from the control data of the fast-freeze plant where fillets of fish are exposed on endless metal belts cooled to —45°C.(—49°F.) by a brine of calcium chloride. The bacterial count

of the fillets is reduced 20.1 per cent by 12 hours at —2.5°C.(27.5°F.) and 58.7 per cent by quick-freezing. Hess (1934) reported that a suspension of marine bacteria, isolated from cod slime, frozen in sea water at —16°C.(3.2°F.) was reduced in number 40 to 70 per cent in eight minutes, and 99.9 per cent after 4.5 hours' freezing.

EXPERIMENTAL PROCEDURE

The method of studying the effect of fast-freezing upon the bacterial flora of mackerel in this laboratory was as follows:

Samples of the flank muscle, which has been shown by Birdseye (1929) to be more highly contaminated than the back muscle, and samples of the intestinal contents were removed and plated in suitable dilution in ZoBell's (1941) agar, which is a medium based on the use of sea water. These were control series. The fish were frozen in a fast-freeze cabinet

TABLE 1

Effect of Fast-Freezing and Storage at Low Temperature Upon Bacterial Flora of Fish After 10 Days

No.	Days stored at —28°C. (—18.4°F.)	Muscle bacteria per gram		Intestinal content, bacteria per gram	
		Before freezing	After freezing	Before freezing	After freezing
1	5	1,400	630	60,000,000	600
2	5	100	6,500	10,500	18,900
3	6	1,190	4,000	3,700,000	440,000
4	7	7,000	2,300	5,000,000	21,000
5	7	250	80	10,000	1,880
6	10	5,700	5,300	1,560	130
7	10	810	480	10,000	6,100
8	10	3,000	70	7,000	2,600
9	10	940	100	3,900	200
10	10	110	40	11,500,000	230,000

by a single-contact process at —28°C.(—18.4°F.) ± two degrees C. and were stored for five to 10 days. They were then removed and additional samples were plated on ZoBell's agar. All plates were incubated six days at room temperature before counting.

The counts of the muscle samples varied rather widely, but in no case was there a count of more than 7,000 organisms per gram, indicating that, bacteriologically at least, the fish were in good condition (Table 1). The decrease in numbers of bacteria in samples stored 10 days was 43.3 per cent. For the increase in sample No. 2, which was treated five days, no certain explanation is offered.

The bacterial content of the intestines was found to vary even more widely than did that of the muscles. This is not surprising since it has been shown by Hunter (1920) that the gut of non-feeding fish is sterile, while the fish having the highest bacterial count were the ones having "chum" (chopped sardines used as bait) in their bellies. Since this "chum" is often in an advanced state of decomposition, the bacterial count is readily explained. The decrease in count of the intestinal content is much more striking than is that of the muscle, but it is probably

less significant since the fish would be eviscerated before use. The average decrease in numbers of bacteria in the samples stored 10 days was 97.9 per cent.

Because of the difficulty of sampling and the possibility of error owing to selection of specimens from different areas, it was decided to isolate some of the typical colonies appearing in largest numbers on the plates and to study the effect of freezing upon relatively dilute suspensions of their organisms.

The cultures used were isolated from the intestines and muscles of mackerel, and were forms which occurred very frequently in the fish examined. Thus they represent an important fraction of the contained flora. All four cultures tested here grew readily at 0°C.(32°F.) but only cocci multiplied at 37°C.(98.6°F.). Of the cultures used No. 20-3 was identified as *Micrococcus varians*, while No. 18-2 is probably *Micrococcus cereus*. Nos. 17-7 and 17-8 are motile, gram-negative, actively proteolytic rods, tentatively classified as Achromobacter sp.

TABLE 2

Effect of Fast-Freezing Upon Suspensions Frozen and Then Stored at —28°C.(—18.4°F.)

Culture No.	Control	After freezing	After 48 hours	Decrease
				pct.
17-7	52,000	72,000	5,100	93.0
17-8	3,200	5,600	1,750	68.8
18-2	29,000	22,000	1,680	92.4
20-3	30,000	60,000	11,500	80.9

The medium used was ZoBell's broth in which the cultures were transferred daily and incubated at room temperature so that they might always be at a similar stage of growth when further tests were performed. Dilutions were made in duplicate in sterile sea water and were plated on ZoBell's agar. All plates were incubated at room temperature for six days before counting.

The dilutions of the cultures were placed in an alcohol bath to freeze and a control thermometer was inserted in a duplicate tube in order to determine the time necessary to reduce the dilutions to the temperature of the bath. This time was determined to be comparable to that which was required to freeze the fish from which the cultures were isolated. Plates were made from one of the tubes when the temperatures of the tubes were within one or two degrees of that of the bath, and again they were prepared after storing for 48 hours at the indicated temperature. The temperature of the bath did not vary more than ± two degrees C.

RESULTS

The effect of freezing and storage at —28°C.(—18.4°F.) on the suspensions (Table 2, second column) indicates a pronounced increase in numbers of all but No. 18-2, which was the slowest growing of the cultures. This apparent exception is probably explained by the fact that the suspensions were not reduced below freezing for a period of one hour after

exposure to the refrigerating bath. The plates made at the end of 48 hours showed that marked reduction in numbers had occurred. This amounted to 93 per cent for 17-7, 68.8 per cent for 17-8, 92.4 per cent for 18-2, and 80.9 per cent for 20-3.

The results of freezing and storage for periods as long as 15 days at —20°C.(—4°F.) show that the effect is, in general, the same as that produced by the lower temperature but is less pronounced at the end of 48 hours (Table 3). The percentage of decrease at this time is 80 per cent for 17-7, 58.4 per cent for 17-8, 35.9 per cent for 18-2, and 14 per cent for 20-3. In seven days 17-7 had decreased 99.5 per cent, 17-8 had decreased 99.9 per cent, 18-2 had decreased 96.8 per cent, and 20-3 showed

TABLE 3

Effect of Fast-Freezing Upon Suspensions Frozen and Then Stored at —20°C.(—4°F.)

Culture No.	Control	After freezing	After 48 hours	After 7 days	After 15 days	Decrease
						pct.
17-7	7,900	10,100	2,020	59	0	100.0
17-8	13,100	19,200	8,000	32	4	100.0
18-2	310	240	154	10	25	91.9
20-3	2,400	2,500	2,150	2,800	1,120	53.4

a count slightly higher than at the time of freezing. In fifteen days the percentages of decrease were as follows: 17-7, 100 per cent; 17-8, 100 per cent; 18-2, 91.9 per cent; and 20-3, 53.4 per cent.

Other experiments were performed in which heavier suspensions were used. The results were similar to those reported here.

DISCUSSION AND CONCLUSIONS

These results differ somewhat from those of Hess (1934), who reported reductions of 40 to 70 per cent in suspensions of marine bacteria frozen for eight minutes at —16°C.(3.2°F.) in sea water and an increase to 99.9 per cent after 4.5 hours. They are in agreement with the results obtained in this laboratory with frozen fish and with the figures quoted by Birdseye (1929) showing reductions of 58.7 per cent by means of quick-freezing fillets of fish.

It is interesting to note that the percentage decreases after 48 hours at —28°C.(—18.4°F.) are greater than the decrease in a similar time at —20°C., inasmuch as the effect of low temperature is not always in direct proportion to the drop in temperature.

Both strains of Micrococci were considerably more resistant to the low temperatures than were the other organisms.

SUMMARY

The bacterial flora of the muscle and intestinal contents of mackerel is reduced sharply by freezing and storage at —28°C. for 10 days.

Freezing and storage for 48 hours at —28°C. produced a greater decrease in numbers in suspensions of bacteria isolated from mackerel than did a similar freezing and storage period at —20°C.

Freezing and storage at —20°C. for 15 days resulted in an approximate 100-per cent decrease in suspensions of Achromobacter sp. used, but the Micrococci withstood the low temperature much better.

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A COMPARISON OF FOUR METHODS FOR DETERMINING VITAMIN C WITH A 25-DAY, WEIGHT-RESPONSE BIOASSAY [1]

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The object of this investigation was to compare several methods for determining vitamin C, including a 25-day, weight-response method of bioassay. At the present time chemical methods have largely replaced bioassay methods for the determination of vitamin C, but where the former are used, investigators frequently find it necessary to check their results against a bioassay method. In instances where it is necessary to make such a check it would be of definite advantage to have a bioassay method available which was simpler and more rapid than the present standard methods.

Within the past two years biologically inactive forms of ascorbic acid, such as d-iso-ascorbic acid and d-gluco-ascorbic acid, have been made available to the food industry and are being used as antioxidants. A bioassay method for determining vitamin C is necessary in the evaluation of the antiscorbutic properties of a foodstuff containing such an antioxidant because chemical methods are not specific for biologically active ascorbic acid. It would thus appear that today there is an increasing need for a simple and accurate bioassay method for vitamin C.

In this paper a short, weight-response, guinea-pig, bioassay method is presented which is essentially that of Coward and Kassner (1936) and Coward (1938) except that it is completed in 25 days instead of six weeks.

BIOLOGICAL ASSAY METHODS FOR VITAMIN C

Of the biological-assay methods for vitamin C, the standard method of Sherman, LaMer, and Campbell (1922) requires 90 days, although numerous workers have reduced the time to 60 days. The curative method of Harris and Ray (1933) and the weight-response method of Coward (1938) both require six weeks for completion. Coward (1938) found the weight-response method to be as accurate as the tooth method of Key and Elphick (1931). Recently Kirk and Tressler (1941) described a 21-day curative method which King found to be as satisfactory as the 90-day preventive method.

In comparison with the above methods the weight-response method described herein is conducted in a period of 25 days. It is based largely on the assumption that guinea pigs should give proportional weight responses to graduated amounts of vitamin C. A second criterion, in addition to the weight response, is the degree of protection from scurvy that the animals show at the end of the test. Since .5 to .6 milligram of ascorbic acid daily prevents guinea pigs from becoming scorbutic, when

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feeding daily supplements containing at least this much vitamin C, one would expect complete protection from scurvy and no scorbutic lesions on dissection of the animals.

DESCRIPTION OF 25-DAY, WEIGHT-RESPONSE METHOD

Healthy 250 to 300-gram guinea pigs growing normally in individual cages were fed various amounts of pure ascorbic acid in aqueous solution for 25 days. During the test the animals were fed the basal Sherman, LaMer, and Campbell (1922) vitamin C-free diet and distilled water *ad libitum*. The levels of ascorbic acid fed ranged from .4 to .8 milligram daily. Response curves, plotting the gain in weight against the amount

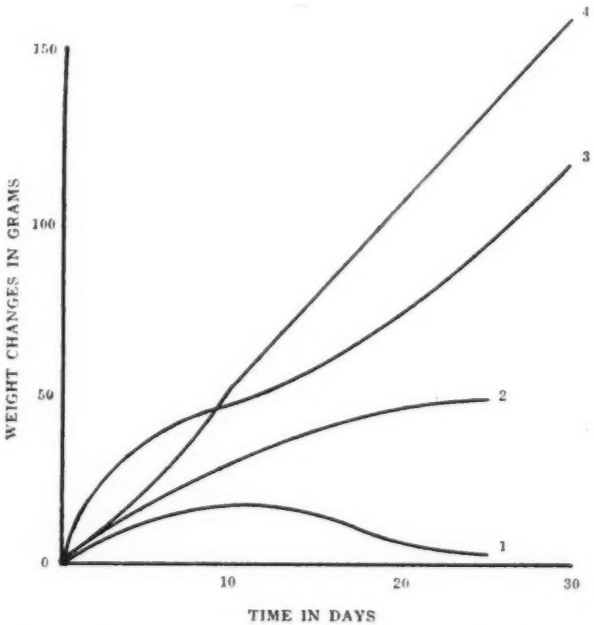

FIG. 1. Average weight-response curves of guinea pigs to graded quantities of ascorbic acid.

Group supplement	Scurvy scores	Number of animals
1. .4 mg. pure ascorbic acid	4.4	10
2. .5 mg. pure ascorbic acid	0	5
3. .6 mg. pure ascorbic acid	0	10
4. .8 mg. pure ascorbic acid	0	10

of ascorbic fed daily, are presented (Fig. 1). Similar response curves were compared with those obtained by feeding verious levels of the food being tested. The food supplement giving a response comparable to a particular control level of ascorbic acid and approximately the same protection from scurvy was considered as containing an equivalent amount of ascorbic acid.

It should be mentioned that in conducting a bioassay by the weight-response method the smallest amount of a supplement giving a desirable response is usually representative of the truest value. A small amount of supplement is used more efficiently by the animal than is a large amount.

The weight-response method of bioassay for vitamin C should give satisfactory results in the hands of any careful technician observing the following precautions:

(a) Be sure that the guinea pigs are young, healthy, and growing at a normal rate.

(b) Begin all animals on the supplementary feeding when they weigh approximately 275 grams.

(c) Use an equal number of animals of the same sex in each different group. In some instances male guinea pigs gain weight slightly more rapidly than do females.

(d) Use at least five or, if convenient, 10 animals on each level.

The amount of vitamin C in the supplement being tested is calculated by comparing the weight response of a test group of animals to a corresponding weight response from a known amount of ascorbic acid fed to one of the control groups of animals, by the following formulae:

$$\frac{\text{Weight gained on supplement}}{\text{Weight gained on known amount of ascorbic acid}} \times \frac{\text{Amount of vitamin C in supplement}}{\text{Amount of vitamin C fed}}$$

EXPERIMENTAL PROCEDURE

Three bioassay and two chemical methods were compared in determining the vitamin C content of canned tomato juice, canned grapefruit juice, fresh spinach, and fresh sweet corn.

The bioassay methods used were the standard method of Sherman, La-Mer, and Campbell (1922), the Harris-Ray (1933) short curative method, and the 25-day, weight-response method. One chemical method used was the 2-6-dichlorophenolindophenol dye-titration method of Tillmans, Hirsch, and Hirsch (1932) as modified by Bessey and King (1933) and by Mack and Kertesz (1936). The other chemical method used was the iodine-titration method of Stevens (1938). Since all of the methods, excepting the weight-response method, are generally known, they will not be described here. The latter is described above. The Sherman bioassay method was considered as the standard method for determining vitamin C in food products and will be used as reference when comparing results obtained by other methods.

The vitamin C content of controlled samples of canned tomato juice, canned grapefruit juice, fresh spinach, fresh sweet corn, and ascorbic acid was determined by the above five methods. Five guinea pigs were used at each feeding level in the bioassay tests except in the Sherman method, when three animals were used. Throughout the feeding tests the samples were analyzed at frequent intervals by both of the chemical tests so that the final results of the dye-titration and iodine-titration methods represent an average of approximately 25 determinations. The results of

TABLE 1

TABLE 1

Experimental Data Obtained in Comparison of Five Methods for Vitamin C Determination

Product	Sherman, LaMer, and Campbell bioassay				Harris-Ray bioassay				Weight-response bioassay (25 days)				Indophenol titration	Iodine titration
	Amount fed	Weight gain	Scurvy score	Ascorbic acid	Amount fed	Weight gain	Scurvy score	Ascorbic acid	Amount fed	Weight gain	Scurvy score	Ascorbic acid	Ascorbic acid	Ascorbic acid
	gm.	*gm.*		*mgm./gm.*	*gm.*	*gm.*		*mgm./gm.*	*gm.*	*gm.*		*mgm./gm.*	*mgm./gm.*	*mgm./gm.*
	....	....	....	....	....	....	....	....	0.4 mg.	2	4.4	....	....	....
Ascorbic acid	....	....	....	....	....	....	....	....	0.5 mg.	50	0.0	....	....	....
	....	....	....	....	....	....	....	....	0.6 mg.	93	0.0	....	....	....
	....	....	....	....	....	....	....	....	0.8 mg.	135	0.0	....	....	....
	2 c.c.	20	9.0	....	2.5 c.c.	36	4.7	....	3.5 c.c.	17	3.8	....	....	....
Tomato juice	4 c.c.	288	0.0	.12-.13	5.0 c.c.	32	1.5	.15	4.0 c.c.	52	0.3	.12	.13	.22
	6 c.c.	235	0.0	....	7.0 c.c.	59	0.0	.14	5.5 c.c.	125	0.0	.12	....	....
	0.0	Loss	16.0	....	0.5 mg.	26	3.2	....	0.5 mg.	62	0.0	....	....	....
Ascorbic acid control	0.4 mg.	160	2.9	....	1.0 mg.	55	0.4	....	0.6 mg.	115	0.0	....	....	....
	0.6 mg.	320	0.0	....	....	....	....	....	....	....	....	....	....	....
	0.8 mg.	350	0.0	....	....	....	....	....	....	....	....	....	....	....
	0.0	Loss	16.0	....	0.9 c.c.	14	5.3	....	0.9 c.c.	70	1.0	....	....	....
	1.0 c.c.	200	5.8	....	1.8 c.c.	37	0.7	.55	1.8 c.c.	100	0.4	.52	....	....
Grapefruit juice	1.5 c.c.	190	2.5	....	3.0 c.c.	71	0.0	....	3.0 c.c.	110	0.0	.34	....	....
	2.0 c.c.	160	0.0	.30	....	....	....	....	....	....	....	....	.34	.38
	3.0 c.c.	332	0.0	....	....	....	....	....	....	....	....	....	....	....
	0.4 mg.	159	2.9	....	0.5 mg.	29	3.2	....	0.5 mg.	53	0.0	....	....	....
Ascorbic acid control	0.6 mg.	310	0.0	....	1.0 mg.	58	0.4	....	0.6 mg.	117	0.0	....	....	....
	0.8 mg.	325	0.0	....	....	....	....	....	....	....	....	....	....	....
Spinach	0.9	113	0.0	.66	1.5	60	1.3	.77	1.0	70	0.0	.66	.66	.77
	1.5	180	0.0	....	....	....	....	....	....	....	....	....		
	0.6 mg.	120	0.0	....	1.0 mg.	51	3.2	....	0.4 mg.	5	4.4	....	....	....
Ascorbic acid control	1.0 mg.	140	0.0	....	....	....	....	....	0.5 mg.	53	0.0	....	....	....
	5.0	16	5.0	....	6.0	44	5.0	.083	5.0	0	3.0	....	....	....
Corn	7.5	195	0.0	.085	9.0	80	1.0	.088	7.5	24	0.0	.073	.09	.22
	10.0	210	0.0	....	....	....	....	....	10.0	25	0.0	....	....	....
	0.6 mg.	230	0.0	....	0.5 mg.	54	4.0	....	0.5 mg.	22	0.0	....	....	....
Ascorbic acid control	....	....	....	....	1.0 mg.	94	0.0	....	0.6 mg.	49	0.0	....	....	....

the above experimental data are presented (Table 1) and summarized (Table 2).

DISCUSSION

The Sherman, LaMer, and Campbell (1922) bioassay method is considered the standard method for determining vitamin C. It may be seen, however, that this method can be used accurately only when the daily vitamin C supplement does not exceed .6 milligram of ascorbic acid (Table 1). Amounts of ascorbic acid over .6 milligram daily give no better protection from scurvy nor do they give significantly different weight gains over the 90-day experimental period. When large quantities of food are fed as a supplement, a serious error may arise, owing to oxidation, if the food remains uneaten in the feed cups for a long period of time.

It is clear that the 2-6-dichlorophenolindophenol dye-titration method gave results in good agreement with the Sherman bioassay method when testing for vitamin C in tomato juice, grapefruit juice, spinach, and corn (Table 2). The weight-response, bioassay method compared favorably

TABLE 2

Summary of Results Obtained in Comparison of Five Methods for Vitamin C Determination

Substance	Bioassay methods			Chemical methods	
	Sherman	Harris-Ray	Weight-response	Indophenol-titration	Iodine-titration
	mgm./gm.	*mgm./gm.*	*mgm./gm.*	*mgm./gm.*	*mgm./gm.*
Tomato juice	.12	.14	.12	.12	.22
Grapefruit juice	.30	.55	.34	.34	.38
Spinach	.66	.77	.66	.66	.77
Corn	.08	.08	.07	.09	.22
Ascorbic acid	.50	....	.50	.50	.50

with the dye-titration method in comparing these two methods with the Sherman test. In most cases the Harris-Ray bioassay method and the iodine-titration method did not check too well with the standard Sherman method.

In general, the 2-6-dichlorophenolindophenol dye-titration method gave results which were in good agreement with the Sherman method. It is seldom that animal-assay results check within 10 per cent with the results obtained by chemical methods, yet in most instances in this investigation these two methods were in even closer agreement. Thus, the 2-6-dichlorophenolindophenol dye-titration method, as used in this investigation, may be considered reliable and accurate in determining the ascorbic acid content of vegetables, plants, citrus fruits, and other common sources of vitamin C.

The Stevens iodine-titration method for vitamin C gave values which were high in most cases. For certain types of routine work on the same product the iodine method might be used to advantage but in general it is not considered as satisfactory as the dye method.

There was a considerable difference in the results obtained in comparing the Harris-Ray method with the Sherman method. In the case

of grapefruit juice there was a difference of 83 per cent. It was also noted that this method is not accurate when small differences in vitamin C content are to be detected. The detection and scoring of incipient scurvy also requires much experience and judgment on the part of the laboratory worker.

With the 25-day, weight-response method the results on the four products tested were in relatively good agreement with those obtained with the standard Sherman method and the dye-titration method. It was also found to be more accurate than the Harris-Ray method or the iodine-titration method. This method requires less time and is simpler to use than the Harris-Ray method, since the major part of the weight-response, bioassay method depends on weight responses which can be accurately measured.

In view of the fact that inactive forms of ascorbic acid, such as d-iso-ascorbic acid and d-gluco-ascorbic acid, are now being used in food products as antioxidants the usual chemical methods for vitamin C determination cannot be relied upon to give a true vitamin C value for such products. In working with such products it is necessary to use a bioassay method in determining their vitamin C content. For this type of work the weight-response, bioassay method should be of particular value because a true picture of the vitamin C content of a product may be obtained without resorting to the time-consuming Sherman method.

Since only scanty data are available on comparative results obtained by the several methods of estimating vitamin C, this preliminary report is presented in the hope that other workers may be interested in using a short, weight-response method of assay.

CONCLUSION

A 25-day, weight-response, bioassay method for determining vitamin C is described and compared with two other bioassay methods and two chemical methods in determining the vitamin C content of canned tomato juice, canned grapefruit juice, fresh spinach, and fresh sweet corn.

Of the different methods compared in this investigation the 2-6-dichlorophenolindophenol-titration method and the 25-day, weight-response, bioassay method gave good checks with the standard Sherman bioassay method. The weight-response, bioassay method provides a simple and accurate method of checking the results of chemical determinations for vitamin C in food products.

The weight-response, bioassay method should be of particular aid in evaluating the antiscorbutic properties of a food product containing added inactive or possibly naturally occurring forms of ascorbic acid or an antioxidant. In such cases the available chemical methods give the total ascorbic acid contents but do not differentiate between biologically active and inactive forms of vitamin C.

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LOSS OF VITAMIN C DURING COOKING OF SUMMER SQUASH

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Two varieties of summer squash, the Early White Bush Scallop or White Patty Pan and the Yellow Crookneck, are commonly found in the diets of Texans and are generally grown over the state. Hence, an investigation of the amount of vitamin C contributed by these to the Texas dietary seems in order. Only one study, that of Burrell and Ebright (1940), reports the vitamin C content of the White Bush squash. Consequently, the purpose of this study is twofold: (1) to determine the vitamin C content of the White Bush and Yellow Crookneck squash available in Denton, Texas, and (2) to determine the effect of different methods of cookery upon the vitamin C in these two varieties of squash.

EXPERIMENTAL PROCEDURE

The samples of the two varieties of summer squash used in this study were obtained from the local market and from a local garden. Three lots of the White Bush were tested: (1) garden fresh; (2) stored in open market four days; and (3) stored in open market eight hours. One of the two lots of Yellow Crookneck squash tested was garden fresh, and the other had been stored in open market for eight hours. The eight hours of storage elapsed from the time the fresh produce was delivered to the store and the opening of the store for early-morning retail trade. The White Bush variety, which had been stored in the open market for four days, was tested to determine the effect of "overstocking" upon the nutritive value of fresh vegetables. The garden-fresh vegetables were gathered early in the morning. All vegetables were stored in covered enamel containers in an electric refrigerator and all were tested late in the afternoon of the day of purchase.

The Yellow Crookneck squash selected was of medium size—approximately five to the pound in weight—and was prepared for cooking by washing and cutting into slices about one-half inch in thickness. All were prepared in the same way at the same time, mixed thoroughly to insure uniformity of samples, and divided into one-pound lots for cooking. Each of the one-pound lots of sliced squash was then cooked by boiling rapidly in identical two-quart, covered, enamel saucepans. Ten minutes was used as a desirable cooking time, since Wilmot and Batjer (1938) suggest eight to 12 minutes for this squash. One-half cup of water was used in cooking Lot 1. Tap water was used throughout the study in order to obtain results which are indicative of the summer squash consumed by the residents of Denton. The second and third lots were cooked in one cup and one and one-half cups of water for 20 and 30 minutes, respectively. The fourth

method employed is one commonly used by Texas housewives, namely, parboiling for three minutes (Lot 4) and sautéing in bacon fat for 10 minutes. One squash weighing three and three-fourths ounces was boiled whole in two cups of water for 20 minutes. The 10-minute cooking period applied to Lot 1 was repeated with another lot to the water of which salt was added, since Fixsen (1939) reports that salt retards the destruction of vitamin C.

The amounts of water used were such that no water remained at the end of the designated cooking periods except in the case of the three-minute parboiled squash. It seemed advisable to use this procedure since housewives are urged to prevent cooking losses and this is an effective means of doing so. The samples tested include the vitamin C of the squash and such vitamin C as remained on the squash after the evaporation of the cooking water. Aliquots of the water from the parboiled squash were added to the sautéed product and the two analyzed for vitamin C at the same time in order to make all the samples comparable.

The White Bush squash was prepared for cooking and cooked in the same manner as the Yellow Crookneck. The only exceptions were the following: (1) cutting the White Bush into one-half inch cubes instead of slicing; (2) boiling a White Bush squash, weighing three and one-half ounces, whole for five minutes and then baking at 176.7°C.(350°F.) for 20 minutes; and (3) repeating (2) but removing the inside as for stuffing, returning this to the cavity, and baking.

Each sample was cooked according to the methods described for the two varieties of squash named. Each lot was cooked in triplicate.

The method of assay used in this study was that described by Bessey and King (1933) and modified by Mack and Tressler (1936). All analyses were made in triplicate by placing the hot aliquots in the acid and grinding at once. The figures herein reported are the average of the three analyses.

DISCUSSION OF RESULTS

The methods employed in cooking the two varieties of squash were with one exception—the boiling of the whole squash—the most commonly used methods in Texas. The effect of cooking on the vitamin C content is especially important because in at least two instances outbreaks of scurvy have been reported on diets containing more than adequate amounts of highly antiscorbutic vegetables (1932). An investigation showed that they were improperly cooked, having been incorporated in meat stews or cooked in large quantities for at least five hours, a period long enough to destroy all the original vitamin C. In the average Texas home the problem of overcooking is a serious one, although cooking for five hours is rare.

It is evident that Sample 2 of Early White Bush, which was stored at room temperature in an open market for eight hours, lost less than Sample 1, which had been kept for four days owing to an overstocking of fresh vegetables, when compared with the freshly gathered (garden-fresh) sample (Table 1). A loss of 43 to 55 per cent of the vitamin C as compared with the garden-fresh sample emphasizes the importance of know-

ing the source of vegetables used in estimating the vitamin C content of the diet. When storage loss is coupled with the loss resulting from the cooking method used, the vitamin C of a good source may become a poor source of that same vitamin. The four-day loss reported here is greater than Tressler and co-workers (1937) reported lost from lima beans which had been stored four days, but about the same amount which they report as lost from spinach stored four days. However, some of the loss in the present study may have been due to the badly bruised condition of the squash when it was delivered, since Kertesz, Dearborn, and Mack (1936)

TABLE 1

Comparison of Amount of Vitamin C in Three Samples of Early White Bush Squash and Percentage Lost by Cooking

Method of cooking	Garden-fresh sample			Open-market sample 1[1]			Open-market sample 2[2]		
	Vitamin C	Amt. lost	Loss	Vitamin C	Amt. lost	Loss	Vitamin C	Amt. lost	Loss
	mg./ 100 gm.	*mg.*	*pct.*	*mg./ 100 gm.*	*mg.*	*pct.*	*mg./ 100 gm.*	*mg.*	*pct.*
Raw	29	0	0	13	0	0	23	0	0
Boiled 10 minutes	17	12	41	8	5	42	14	9	39
Boiled 10 minutes in salted water	18	11	39	8	5	38	15	8	40
Boiled 20 minutes (overdone)	14	15	53	6	7	56	10	13	57
Boiled 30 minutes (overdone)	13	16	56	5	8	61	9	14	62
Boiled whole 20 minutes (tender)	17	12	43	7	6	45	13	10	44
Boiled 5 minutes, baked 20 minutes (tender)	10	19	67	5	8	67	8	15	65
Boiled 5 minutes, stuffed and baked 20 minutes (tender)	9	20	70	4	9	72	6	17	73
Boiled 3 minutes, sautéed in bacon fat 10 minutes	7	22	76	3	10	76	5	18	77

[1] Stored in open market at room temperature four days. [2] Stored in open market eight hours.

found that the breaking down of cell walls and subsequent oxidation of the vitamin caused a loss of vitamin C.

The data (Table 1) also show that the loss of vitamin C in garden-fresh squash increased with cooking time—41, 53, and 56 per cent for 10, 20, and 30 minutes' boiling, respectively. Boiling in salted water did not appreciably improve the retention of vitamin C since salted water gave a loss of 39 as compared with 41 per cent for the same boiling period. Boiling the vegetable whole for 20 minutes decreased the loss to approximately that of the cubed squash which was boiled for only 10 minutes. When Early White Bush squash is parboiled for five minutes prior to stuffing and baked for 20 minutes, the loss—70 as compared with 67 per cent—is somewhat similar whether the skin is broken, as for stuffing, or not. However, boiling for three minutes and then sautéing for 10 minutes,

the most destructive method employed in the cooking of summer squash by the Texas housewife, results in a 76-per cent loss. The analyses of the squash samples used in the present study include the liquid in which they were cooked since a minimum of water was used in the beginning. The vitamin C value reported by Burrell and Ebright (1940) compares favorably with the higher market value obtained in this study, namely, 23 milligrams of vitamin C per 100 grams of the raw vegetable.

It is evident that the percentage loss for all samples of Early White Bush squash is similar except for the 30-minute boiling period, when the percentage of the vitamin C loss is greater for both of the open-market samples than for the garden-fresh, namely, 61 and 62 as compared with 56 per cent (Table 1).

TABLE 2

Comparison of Amount of Vitamin C in Two Samples of Yellow Crookneck Squash and Percentage Lost by Cooking

Method of cooking	Garden-fresh sample			Open-market sample		
	Vitamin C	Amt. lost	Loss	Vitamin C	Amt. lost	Loss
	mg./ 100 gm.	*mg.*	*pct.*	*mg./ 100 gm.*	*mg.*	*pct.*
Raw	30	0	0	22	0	0
Boiled 10 minutes (tender)	17	13	43	13	9	40
Boiled 10 minutes in salted water	18	12	40	14	8	39
Boiled 20 minutes (overcooked)	14	16	53	11	11	49
Boiled 30 minutes (overcooked)	13	17	56	10	12	53
Boiled whole 20 minutes	17	13	42	13	9	43
Boiled 3 minutes, sautéed 10 minutes in bacon fat	7	23	72	6	16	72

A comparison of Tables 1 and 2 shows insignificant differences in the vitamin C content of the Early White Bush and the Yellow Crookneck squash. The addition of salt to the boiling water before the squash is added has little effect on the vitamin C content of either variety. A critical analysis of the two tables shows conclusively that the 10-minute boiling period, with or without salt, is the best method to use to prevent vitamin C loss in White Bush and Yellow Crookneck squash. From the standpoint of vitamin C conservation, sautéing, perhaps the most commonly used method of cooking squash in Texas, is the least desirable of the cooking methods used in this study.

If each Texan depending on his general food supply for the 60-milligram minimum vitamin C daily requirement for the adult man or woman, King (1938), or the 75-milligram optimum, National Research Council (1941), eats four ounces (one-half cup) of garden-fresh White Bush or Yellow Crookneck squash boiled in either salted or unsalted water for not longer than 10 minutes, he gets approximately one-third to one-fourth of his daily requirement of vitamin C. On the other hand, if he eats the same kind of squash which has been sautéed, it will be necessary for him to eat approximately one and one-half cups to get one-third of his daily requirement of vitamin C.

SUMMARY

Summer squash, White Bush and Yellow Crookneck varieties, loses less vitamin C with increased boiling time when the samples are garden fresh.

A 10-minute boiling period using a minimum amount of salted or unsalted water destroys the least vitamin C, namely, 40 per cent.

The method of parboiling and then sautéing destroys more vitamin C than any other method tested, about 72 to 77 per cent.

One-half cup of either the White Bush or the Yellow Crookneck squash cooked for 10 minutes in boiling water will furnish one-fourth to one-third of an adult's daily vitamin C requirement.

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FACTORS INFLUENCING THE TEXTURE OF PEAS PRESERVED BY FREEZING [1]

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With the rapid growth of the pea-freezing industry, there has been need for more information regarding the variations in texture of frozen peas and the causes of such variations in order to eliminate or control, if possible, those factors which contribute to toughness or lack of uniformity in the pack. The specific texture investigations reported in this paper are as follows: (1) the amount of variation for a given sieve size and variety of peas grown in the same field and harvested on the same day; (2) variations for a given sieve size and variety of peas grown in the same field but harvested on different days of the same season; (3) effect of cooking for 5, 10, and 15 minutes; (4) effect of vining; and (5) effect of delay between vining and freezing.

The texture of the skins was determined by measuring the load required to penetrate the skins, and the texture of the cotyledons was determined by measuring the load required to crush cotyledons to one-fourth of their thickness. Measurements were made on cooked peas.

Gowen (1928), in an investigation with canning peas, showed that during delay calcium and magnesium content of the skins increase and the skins toughen. Sayre, Willaman, and Kertesz (1931), reporting on canning peas, found that machine-shelled peas have higher puncture and crushing values than hand-shelled ones and that the difference is far more marked in small sizes than in large peas. Their tests were made on whole, uncooked, fresh peas, and they state that the tests are not satisfactory with cooked peas. They found that puncture values increase with time after shelling, indicating that the skin becomes tougher; this was true for both hand-shelled and machine-shelled peas. The crushing test did not change in either direction consistently when peas were held after shelling. They explain that this is because the crushing value is affected by two factors: the firmness of the pulp, which is probably decreasing, and the toughness of the skin which is increasing owing to migration of calcium and phosphorus. They also state that size alone is not always an index to texture. Kohman and Sanborn (1936) also report that canned peas are tougher,

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particularly the skins, in proportion to the holding period after vining. They found that hand-shelled peas held for the same time under the same conditions as viner-shelled peas do not show this toughening effect. Further, they found that both the cotyledons and the skins increase in calcium content while being held after vining; they suggest that since leaves are generally high in calcium in comparison with other plant tissues, the leaf and vine juice may furnish a source of calcium to be absorbed by the peas during the holding period after vining. Campbell and Diehl (1937), basing their conclusions on organoleptic tests of frozen peas, state that raw, viner-shelled Alderman peas may be held for six hours at 21.1°C.(70°F.) or for 24 hours, if covered with crushed ice, without significant deterioration in texture.

EXPERIMENTAL PROCEDURE

The methods employed in growing and harvesting the peas, except as otherwise noted, were comparable to those followed in commercial production of frozen peas in Washington. The soil, a silt loam, of good depth and average fertility was thoroughly prepared by plowing, disking, and harrowing. Approximately 300 pounds of 18-per cent superphosphate per acre was drilled with the seed. The seed was drilled at approximately 250 pounds per acre for all varieties except tall Alderman, for which the rate was increased to approximately 275 pounds per acre. The plots were dusted once with rotenone for pea-weevil control. Otherwise, they were grown without additional treatment until harvest. The plots were mowed with standard equipment and vined within one hour with a Hamachek Ideal viner. Experimental field-run samples were obtained directly from the viner hopper.

The freezing procedure in 1937 and 1938 consisted of cleaning, scalding at 98.9°C.(210°F.) for one minute, cooling in running water, packing in No. 2 plain tin cans, hermetically sealing, and immediately storing in a room held at —17.8°C.(0°F.). (Analysis of the water used for cleaning, scalding, and cooling the peas is not available, but the water is commonly used in the batteries of automobiles and therefore must be low in minerals.) A change in freezing procedure was made in 1939. The peas packed that season were frozen on a tray in a freezer held at —28.9°C. (—20°F.) and as soon as the peas were solidly frozen, they were packed in tin cans and stored as before at —17.8°C.(0°F.). During the 1939 packing season the tenderometer reading of all peas was taken. The tenderometer, which was developed by Martin (1937), measures the load required to shear a four-ounce sample of raw peas. The tenderometer is used for establishing the price paid for canning and freezing the peas. The reading for this purpose is taken after the peas are cleaned but before they are scalded.

After several months of storage the frozen peas were removed from the —17.8°C.(0°F.) freezer and immediately graded for size according to the standards for canning peas, United States Department of Agricultur (1931). Preliminary trials had shown that approximately 97 per cent of the solidly frozen peas graded the same size as fresh peas. After size grading, the peas of all except one sieve size were packaged and returned to storage for a maximum of four days until texture measurements could

be made. Peas of the one size were cooked by placing them in an equal weight of boiling, unsalted, distilled water and cooking them in an uncovered utensil for six minutes after the water reached the boiling point. At the end of the cooking period the peas were drained, placed one layer deep in a wire sieve, and air-cooled in room temperature for 10 minutes before texture measurements were made.

The apparatus used for the texture measurements were developed by Candee and Boggs (1941) at the State College of Washington. For the peas packed in 1937 and 1938, the texture of both the skins and the cotyledons was measured, that of a cotyledon by determining the load in kilograms required to crush a cotyledon of a cooked pea, from which the skin was removed, to one-fourth of its thickness. The texture of skins was measured by determining the load required to penetrate three layers of skins of cooked peas from which cotyledons were removed with a one-eighth inch, ball-bearing penetration point.

By using two plungers, one with a penetration point and the other with a crushing disc, the same apparatus could be used for both the skin and the cotyledon measurements. The load was applied by allowing shot to flow into a weight-pan, which rested on the plunger to which the penetration point or crushing disc was attached. After each test, the weight-pan was removed and weighed. The load was applied at the rate of approximately 2.8 kilograms per minute. Fifty readings for skins or fifty readings for cotyledons, each from peas of one sieve size, were averaged to obtain the penetration value or the crushing value for the peas of one size.

In 1939, only the texture measurements of skins were made and a different apparatus was used. The change in emphasis to skin texture only was made for several reasons. The importance of skin-texture measurements was recognized. The number of problems to be investigated was large. Some of the problems with cotyledons were being investigated in connection with the establishment of the tentative grades for frozen peas by the Agricultural Marketing Service of the U. S. Department of Agriculture (1939). Since cotyledon measurements were to be discontinued, at least temporarily, the mechanism for measuring cotyledon height was no longer needed and a simple apparatus was developed by Candee and Boggs (1941). It differed in the method of application of load, which was accomplished by moving a weight away from the fulcrum of an arm and toward the penetration point attached to the other end of the arm. By this method, the load could be read directly from the arm, and measurements could be made more rapidly. Beginning with the peas packed in 1939 the number of skin measurements for peas of Sizes 5, 6, and 7 was increased to 100. Although the general method of testing skins with the two apparatus was the same, a conversion factor was not obtained. Therefore, results in one table of this paper should not be compared with those of a different table, unless the taste grades which accompany each table show that the results of the two series are on the same basis.

Mechanical tests of texture of food products are of limited value unless they are related to tasters' judgments of quality. Therefore, taste scores for the texture of peas of known penetration and crushing values were

obtained. The scoring group for each series consisted of four to six research workers, all of whom had had several years of experience in scoring both experimental and commercial packs of frozen peas. The texture of skins and cotyledons of the cooked peas were scored separately. A grade of A for either skins or cotyledons meant that scorers considered those skins or cotyledons to be of highest market quality; Grade B skins or cotyledons were considered marketable but poorer in texture than Grade A, and Grade C were considered too tough to be marketed. The relation between taste scores and penetration or crushing values should not be considered final judgments but they should help the reader in interpreting variations in mechanical values. The taste results which apply to a particular study are listed in the tables to which they apply.

RESULTS AND DISCUSSION

Sieve Size of Peas as an Index of Texture: Sieve size has been used widely as an index of the texture or the maturity of peas. It has also been used to some extent as a means of separating overmature peas from commercial packs. Preliminary work of the writers had indicated that there were very large variations in texture of peas of one sieve size, even when they were of the same variety, grown in the same field, and harvested on the same day. In view of these observations, the reliability of sizing as a means of eliminating overmature peas from the pack was questioned and, therefore, further information regarding the degree of variation for a given sieve size of peas as compared with variations for different sieve sizes in the field-run samples was obtained.

In the first investigation which was conducted in 1938, cooked, fresh peas were used. The variation in texture of a given sieve size was related to the appearance of the pod from which the peas were taken. The varieties of peas studied were Improved Gradus, Tall Alderman, and Thomas Laxton. The peas were grown in trellised rows and were hand-picked and hand-shelled. All of the peas of one variety were picked from the same field on the same day. The pods were divided into three maturity groups on the basis of the physical characteristics associated with advancing maturity. Pods classed as "least mature" were incompletely filled, all green in color, and free from wrinkling or indentation along the sutures; those classed as "more mature" were full but not tight, showed slight color change to gray-green or yellow-green, and slight indentation along the sutures; and those classed as "most mature" were tightly filled, gray-green or yellow-green in color, slightly wrinkled, and indented along the sutures. The classification thus arrived at was arbitrary but was adequate for the purpose of the experiment.

The exact proportion of pods at each stage of maturity was not determined, but on the basis of volume of pods approximately one-fourth were "least mature," one-half were "more mature," and one-fourth were "most mature." Only the peas of sieve Sizes 5, 6, and 7 of each maturity group were tested because these were the only sizes which existed in sufficient numbers in two of the three samples to be compared. This series is the only one in this paper for which fresh, unfrozen peas were used. The

penetration and crushing values for the cooked peas are summarized (Table 1).

Large variation is noticeable in both cotyledon and skin texture of peas of one sieve size for a given variety harvested from the same field at the same time (Table 1). The variations in texture values for peas of different size are less than the variation of one size taken from pods which appear to differ in maturity. This should not be interpreted as meaning that sieve size bears no relationship to texture, because all of the texture tests reported in this paper indicate that the average penetration or crushing value for all of the Size 7 peas of a field-run sample is higher than

TABLE 1

Pod Appearance and Pea Size as Indices of Texture of Cooked, Fresh Peas

Variety	Sieve size of peas	Penetration values and their standard deviations						Crushing values and their standard deviations					
		Least mature		More mature		Most mature		Least mature		More mature		Most mature	
		kg.	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*
Improved Gradus	5	.18	.03	.25	.04	.64	.13	.61	.04	0.68	.12	1.24	.26
	6	.29	.04	.33	.09	.67	.15	.65	.11	0.73	.11	1.25	.34
	7	....	....	.40	.10	.75	.14	....	....	0.88	.17	1.26	.39
Tall Alderman	5	.21	.04	.39	.08	....	....	.72	.14	0.89	.20	....	....
	6	.31	.06	.40	.08	.68	.14	.79	.16	1.02	.19	1.37	.39
	7	.34	.11	.56	.12	.69	.14	.84	.24	1.18	.33	1.41	.44
Thomas Laxton	5	.26	.07	.35	.09	.67	.12	.69	.12	0.72	.12	0.99	.26
	6	.38	.10	.47	.14	.77	.10	.72	.10	0.79	.15	1.32	.35
	7	....	....	.57	.12	.92	.13	....	....	0.95	.31	1.34	.49

TASTE RESULTS RELATED TO TEXTURE VALUES

Tasters' grade	Penetration value	Crushing value
A	.35 kg. or less	.78 kg. or less
B	Not exceeding .51 kg.	Not exceeding 1.13 kg.
C	.54 kg. or more	1.19 kg. or more

the average value for Size 6's of the same sample and the average for all of the Size 6's is higher than the average value for the Size 5's. The results do show, however, that some of the small peas in the field-run sample are much tougher than some of the large peas; therefore, too much reliance should not be placed on sizing as a means of separating peas of different textures. Thus, it appears that a practical method other than sizing is needed for separating tough peas from a field-run sample.

The reactions of consumers to only a few tough peas in a spoonful cannot be estimated, but it seems to the writers, judging from experience in scoring hundreds of such samples, that the presence of even a few tough peas in a taste sample gives an unfavorable reaction to the entire sample.

A second investigation was made in 1939 for the purpose of obtaining information about texture variability within a given sieve size of peas of a given variety grown in the same field but harvested on different days. The tenderometer reading of the field-run sample for each day's harvest

was also taken. Sufficiently large fields of Tall Alderman and Thomas Laxton peas were planted to obtain samples of each on several successive dates. As soon as the peas were vined, the tenderometer test on a field-run sample was made under standard conditions of temperature. At least three tenderometer readings were obtained for each sample, and the average of all readings is reported. The peas were frozen by the 1939 procedure, and after four months of storage, the penetration measurements of skins were made (Table 2).

The penetration values (Table 2) show that for both varieties of peas, as the tenderometer reading of the field-run sample increased, or as the peas were left in the field longer, the average penetration value for a given sieve size of peas increased; this increase was accentuated in the case of the larger peas. In other words, the skin texture of any given sieve size of peas is not constant for a given variety, field, and season; the skins become tougher when the peas are left in the field longer. This applies to each size of peas in the field; for example, the skins of Size 7 Tall Alderman peas cut when the tenderometer reading was 102 were more tender than the Size 6's cut one day later when the tenderometer reading was 118; the skin texture of Size 6 Thomas Laxton peas cut when the tenderometer reading was 90 was approximately the same as the Size 5's from a sample which was cut when the tenderometer reading was 117. Considering the taste grades in relation to their penetration values, the skins of Thomas Laxton peas of Size 5 or 6, or the Tall Alderman peas of Size 5, 6, or 7 were excellent quality or too poor to be marketed, dependent on the number of days they were left in the field. There was a small inconsistency in the tenderometer readings for the Thomas Laxton peas harvested on the 12th and 13th of July. Also, there were some small inconsistencies in the penetration values. For example, Size 7, peas of the Thomas Laxton variety harvested on the 11th of July had a penetration value of 1.43, and on the 12th of July it was 1.36. An increase in the value for the later harvest data would be expected. Nevertheless, the data clearly show that there is a tendency for the penetration values for peas of a given size to increase when harvested on successive dates. Definite "cut-offs" for Grades A, B, and C should not be interpreted from Table 2, because skin texture was the only quality factor investigated; the results were based on one harvest, and six tasters are inadequate for such a conclusion.

Period of Cooking: Toughness of skins of frozen peas would not be a serious defect if cooking would correct it. In order to determine whether cooking for the longest period consistent with acceptable color and flavor retention would yield satisfactory tenderness, samples of peas with varying skin textures were cooked for 5, 10, and 15 minutes. Packs B for 1937 and 1938 (Table 3) were commercially packed and their history is not known. They were selected for this investigation because they were known to be tougher than most commercial samples. All of the other packs reported in Table 3 were experimental samples, and the handling procedures for the different years were previously described.

In approximately one-third of the trials (Table 3) the penetration values of the peas cooked for 10 and 15 minutes were approximately the

TABLE 2

Penetration Values of Skins Related to Tenderometer Readings of Field-Run Samples of Peas Harvested on Different Days

Variety	Date of harvest, July, 1939	Tenderometer reading	Penetration values and their standard deviations										Calculated composite
			Size 7		Size 6		Size 5		Size 4		Size 3		
		lb./sq. in.	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*	*SD*	*kg.*
Tall Alderman	15	76	0.88	.13	0.84	.11	0.75	.08	0.65	.07	.57	.06	0.75
	18	92	1.20	.10	1.05	.18	0.88	.13	0.74	.10	.64	.11	0.98
	20	102	1.43	.24	1.17	.26	0.95	.17	0.84	.16	.65	.14	1.13
	21 a.m.	118	1.71	.32	1.57	.33	1.26	.29	0.90	.19	.73	.14	1.45
	21 p.m.	122	1.90	.35	1.67	.35	1.43	.34	1.10	.28	.84	.21	1.56
Thomas Laxton	11	85	1.43	.31	1.12	.21	0.97	.16	0.77	.14	.69	.12	0.97
	12	90	1.36	.28	1.16	.19	0.98	.14	0.82	.10	.73	.09	1.02
	13	88	1.41	.33	1.23	.26	0.97	.12	0.87	.12	.72	.09	1.06
	14	98	1.53	.34	1.30	.27	1.09	.17	0.98	.20	.82	.12	1.17
	15	100	1.45	.32	1.32	.31	1.11	.22	0.97	.20	.81	.15	1.23
	17	117	1.60	.15	1.48	.32	1.08	.21	1.05	.27	.99	.19	1.32
	19	124	1.69	.29	1.65	.26	1.46	.22	1.07	.24	.92	.15	1.51

TASTE RESULTS RELATED TO PENETRATION VALUES

Tasters' grade	Penetration value
A	1.15 kg. or less
B	Not exceeding 1.35 kg.
C	1.42 kg. or more

same as the values for the same lot of peas cooked for five minutes; in another one-third of the trials the penetration values for the longer cooking periods were actually higher than those for the five-minute cooking period. A considerable decrease in penetration values resulted from the longer cooking periods for the other one-third of the trials. However, in the case of only one of the 13 samples whose skins would have a taste-grade of C when boiled for five minutes would the grade become B when the peas were boiled for 10 or 15 minutes. The lack of uniformity of response to longer cooking periods could not be related to any factor in

TABLE 3

Variations in Skin Texture of Frozen Peas Cooked for 5, 10, and 15 Minutes

Variety	Year packed	Lot	Tenderometer reading	Sieve size	Penetration values		
					Boil 5 min.	Boil 10 min.	Boil 15 min.
			lb./sq. in.		*kg.*	*kg.*	*kg.*
Laxtons' Progress	1937	A	.....	5	.22	.18	.19
	1937	A	.....	6	.29	.27	.25
	1937	A	.....	7	.46	.43	.34
	1937	B	.....	5	.62	.64	.57
	1937	B	.....	6	.72	.85	.81
	1937	B	.....	7	.85	.87	.89
Thomas Laxton	1938	A	.....	7	.36	.27	.19
	1938	B	.....	4	.61	1.00	.93
	1938	B	.....	5	.77	.90	.96
Tall Alderman	1939	A	103	5	.75	.73	.66
	1939	A	103	6	1.09	1.24	1.14
	1939	A	103	7	1.65	1.63	1.47
	1939	B	108	5	.87	.88	.88
	1939	...	108	6	1.67	1.10	1.16
	1939	...	108	7	2.09	1.31	1.45
	1939	C	113	5	1.21	.71	.72
	1939	C	113	6	1.87	1.02	.97
	1939	C	113	7	2.32	1.26	1.37

TASTE RESULTS RELATED TO PENETRATION VALUES

Grade	Penetration values, 1937-38	Penetration values, 1939
A	.35 kg. or less	.48 kg. or less
B	Not exceeding .51 kg.	Not exceeding .81 kg.
C	.54 kg. or more	.88 kg. or more

the known history of the samples. It appears on the basis of this investigation that cooking periods of 15 minutes, the longest period which yields acceptable flavor and color, cannot be relied upon to produce tender skins of frozen peas if toughness is characteristic of the uncooked product.

Effect of Vining and Delay in Handling: Although considerable research has been conducted to determine the effects of mechanical removal of peas from their vines and pods (vining) and delay between vining and processing on canning peas, few studies with frozen peas have been made. Two studies were undertaken, therefore, one in 1938 and the other under slightly different conditions in 1939.

For the 1938 series, peas of the Tall Alderman variety were used. Following vining, one sample was frozen immediately, and the remainder of the peas were divided into three lots: one lot was held at room temperature; one was heavily covered with cracked ice; and the third lot, after being thoroughly washed in running soft water in order to remove the vine and leaf juice, was held at room temperature. An additional sample from the same field was hand-picked at the same time and was hand-shelled. As was the case with the vined peas, one lot of the hand-shelled peas was frozen as soon as shelled and the others were held at room temperature. The periods of delay between shelling and freezing of the

TABLE 4

Effect of Vining and Delay on Skin and Cotyledon Texture of Frozen Peas, 1938

Handling conditions[1]	Penetration values				Crushing values			
	Size 7	Size 6	Size 5	Size 4	Size 7	Size 6	Size 5	Size 4
	kg.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
Hand-shelled, no delay	.45	.36	.28	.20	0.87	0.74	.66	.52
Hand-shelled, 4-hr. delay at room temp.	.53	.45	.37	.23	0.86	0.70	.67	.58
Vined, no delay	.57	.41	.35	.24	1.28	1.03	.85	.66
Vined, 2-hr. delay at room temp.	.62	.54	.42	.32	1.09	0.94	.79	.64
Vined, 4-hr. delay at room temp.	.77	.66	.53	.42	1.16	0.96	.81	.69
Vined, 8-hr. delay at room temp.	.74	.68	.56	.45	1.25	1.04	.82	.57
Vined, iced, 4-hr. delay	.68	.57	.42	.32	1.18	0.93	.76	.62
Vined, iced, 8-hr. delay	.69	.65	.50	.44	1.24	1.08	.80	.68
Vined, iced, 24-hr. delay	.84	.79	.71	.61	1.28	1.01	.75	.56
Vined, washed, 2-hr. delay at room temp.	.67	.53	.38	.29	1.12	0.91	.78	.61
Vined, washed, 4-hr. delay at room temp.	.74	.59	.48	.38	1.26	1.08	.74	.59
Vined, washed, 8-hr. delay at room temp.	.74	.67	.63	.52	1.22	0.99	.85	.70

[1] Peas were washed after delay periods unless exception is noted.

TASTERS' JUDGMENT OF QUALITY RELATED TO TEXTURE VALUES

Tasters' grade	Penetration value	Crushing value
A	.35 kg. or less	.78 kg. or less
B	Not exceeding .51 kg.	Not exceeding 1.13 kg.
C	.54 kg. or more	1.19 kg. or more

peas are listed (Table 4); during the delay periods, the peas were held in 30-pound wooden lugs.

The temperature at the center of the lugs of unwashed, vined peas and that for the hand-shelled ones was 17.8°C.(64°F.) at the beginning of the delay periods; after two hours and again after four hours of delay the temperature was 20°C.(68°F.) and after eight hours it was 22.8°C. (73°F.). Washing the peas in water to remove the vine juice reduced the temperature to 14.4°C.(58°F.), but after two hours the temperature of the washed samples was the same as that of the unwashed ones. The temperature at the center of the lugs of iced peas did not change during the first two hours; after four hours the temperature was 4.4°C.(40°F.); and after eight hours it was 6.7°C.(44°F.). In view of the slow rate of

temperature decrease when the lugs of peas were iced at the surface only, it would seem desirable when icing peas to mix the crushed ice throughout the sample.

At the end of delay periods each sample was frozen in the usual way, and after five months of storage the penetration and crushing tests were made (Table 4).

The standard errors of the differences of means were calculated according to Snedecor (1938) in order to more readily compare certain penetration and crushing values (Table 4). The levels of the (t) values are summarized (Table 5). A (t) value greater than the one-per cent level is considered highly significant; a (t) value between the five-per cent and one-per cent levels is considered significant; and a (t) value less than the five-per cent level is considered not significant.

Vining was shown to be the only handling procedure investigated which consistently and significantly changed the crushing values of the cotyledons of frozen peas (Tables 4 and 5). Cotyledons of vined peas had significantly higher crushing values than those for hand-shelled peas, both when the peas were frozen without delay and when each was held at room temperature for four hours between shelling and freezing. On the basis of the taste results, the cotyledons of the Size 6 and 7 peas were one grade lower after vining.

The results regarding the effect of delay on cotyledon texture were less consistent than those regarding the effect of vining. The crushing values for most of the sizes decreased during the early hours of delay, that is, the cotyledons became softer, but there was no consistent trend for the later delay periods.

The effects of the various handling procedures on skin texture (Tables 4 and 5) may be discussed more simply if the term "tougher" is used instead of "a significant increase in penetration values," and the taste scores seem to justify this. The skins of vined peas of all sizes were tougher than those of similar hand-shelled peas, both when the peas were frozen as soon as they were shelled and when each was held at room temperature for four hours. Skins of vined peas toughened during the first two hours of delay at room temperature and again during the second two hours of delay. The skins of the vined peas which were washed before delay periods also toughened during the first two hours and again during the second two hours of delay. The iced sample was not tested after the second hour, but the skins of all sizes toughened after four hours. Thus, the skins of hand-shelled and vined peas toughened during the first four hours of delay by all of the handling procedures investigated, but the degree of toughening differed for the different handling procedures. The results for the delay between the fourth and eighth hours were less consistent. There was no toughening for any of the sizes of unwashed peas between the fourth and eighth hours, when the peas were held at room temperature, but most of the sizes of peas in the iced lot and in the lot washed before delay toughened between the fourth and eighth hours. The skins of the iced sample toughened again between the eighth and 24th hours. Delay toughened the skins of hand-shelled peas as well as those of vined peas. As previously mentioned, toughening of skins

TABLE 5

Significance of Differences of Mean Texture Values of Table 4

Handling conditions compared [1]		Level of t value for different sieve size					
		Penetration results			Crushing results		
1 [2]	2	t greater than 1% level	t greater than 5%, less than 1% level	t less than 5% level	t greater than 1% level	t greater than 5%, less than 1% level	t less than 5% level
		size	*size*	*size*	*size*	*size*	*size*
Vined, no delay	Hand shelled, no delay	4, 5, 6, 7	.....	.....	4, 5, 6, 7	.....	.....
Vined, 4-hr. delay	Hand shelled, 4-hr. delay	4, 5, 6, 7	.....	.....	4, 5, 6, 7	.....	.....
Hand shelled, 4-hr. delay	Hand shelled, no delay	5, 6, 7	4	.....	4	.....	5, 6, —7
Vined, 2-hr. delay	Vined, no delay	4, 5, 6	.....	7	—7	—5, —6	—4
Vined, 4-hr. delay	Vined, 2-hr. delay	4, 5, 6, 7	.....	.....	.....	4	5, 6, 7
Vined, 8-hr. delay	Vined, 4-hr. delay	.....	.....	4, 5, 6, —7	—4	6, 7	5
Vined, iced, 4-hr. delay	Vined, no delay	4, 5, 6, 7	.....	.....	—5, —6	—7	—4
Vined, iced, 8-hr. delay	Vined, iced, 4-hr. delay	4, 5, 6	.....	7	6	4	5, 7
Vined, iced, 24-hr. delay	Vined, iced, 8-hr. delay	4, 5, 6, 7	.....	.....	4	.....	5, —6, —7
Vined, 4-hr. delay	Vined, iced, 4-hr. delay	4, 5, 6, 7	.....	.....	.....	—4	—5, 6, —7
Vined, 8-hr. delay	Vined, iced, 8-hr. delay	.....	5, 7	4, 6	—4	.....	5, —6, 7
Vined, 2-hr. delay	Vined, washed, 2-hr. delay	4, 6	5	—7	.....	.....	4, 5, 6, 7
Vined, 4-hr. delay	Vined, washed, 4-hr. delay	6	4, 5	7	4	—6, —7	5
Vined, 8-hr. delay	Vined, washed, 8-hr. delay	—4, —5	.....	6, 7	7	.....	—4, —5, 6
Vined, washed, 4-hr. delay	Vined, no delay	4, 5, 6, 7	.....	.....	—4, —5	.....	6, 7
Vined, washed, 8-hr. delay	Vined, washed, 4-hr. delay	4, 5, 6	.....	7	4, 5	6	7
Vined, washed, 8-hr. delay	Vined, 4-hr. delay	4, 5, 6	.....	7	4, 5	—6	7
Vined, washed, 4-hr. delay	Vined, iced, 4-hr. delay	.....	4, 5, 7	6	6	7	—4, —5
Vined, washed, 8-hr. delay	Vined, iced, 8-hr. delay	4, 5	7	6	.....	—6	4, 5, —7

[1] Peas were washed after delay periods and delay was at room temperature unless exception is noted. [2] Column 1 has the higher values unless the sieve size in the analysis column is preceded by the minus sign.

occurred during delay when the peas were washed before delay and when the peas were iced during delay as well as by the usual procedure, namely, delay of unwashed material at room temperature. The rate of toughening for the iced and washed samples, however, was different from that for the usual procedure. First, comparing the effect of icing with the usual procedure, it may be noted that for the four-hour delay sample, all sizes of iced peas were more tender than those held at room temperature, but when the delay period was eight hours the results were inconsistent. Second, comparing the effect of washing the peas before delay periods with the usual procedure, when both samples were held at room temperature, it may be noted that for both the two-hour and four-hour delay periods, the washed peas of Sizes 4, 5, and 6, but not the 7's, were more tender than the peas handled by the usual procedure and again the results for the eight-hour sample were inconsistent. It should be recalled that the washed peas were a few degrees (6°F.) cooler than the unwashed ones at the beginning of the delay period and that the two samples were not the same temperature until approximately two hours later. Now, comparing the effectiveness of icing with washing before delay, it may be noted that icing was more effective in preventing toughening than was washing before delay followed by delay at room temperature. In the previous discussion, it was mentioned that there was considerable inconsistency of results for the samples delayed for eight hours. It is possible that desiccation of the peas during this long period influenced the results, especially since the sample was reduced by this time to approximately eight pounds of peas spread out in a 30-pound lug, and it was observed that the peas appeared dry and some were cracked, whereas the samples handled after four hours had appeared moist.

Two of the findings regarding the effect of delay on skin texture appear to differ from those of other workers, although in each case the method or the material was quite different from those of this investigation. Campbell and Diehl (1937) reported that a delay period of 24 hours under ice produced no significant effect upon the texture of frozen peas. However, it may be pointed out that the findings reported by these authors were based upon taste scoring of raw, defrosted, frozen peas rather than upon cooked samples and that under such conditions, it is possible that differences in skin texture are less pronounced than when the peas are cooked. Furthermore, at the time of this particular work the importance of skin texture as a quality factor in frozen peas was not fully realized and, therefore, emphasis was placed on cotyledon texture rather than skin texture. The present findings regarding toughening of skins of hand-shelled peas owing to delay appear to disagree with a report by Kohman and Sanborn (1936). They stated that canned peas are tougher, particularly the skins, in proportion to the holding period after vining, but that hand-podded peas held for the same time under the same conditions as viner-shelled peas do not show this toughening effect. It is possible that the period of heat processing used for canning made a small change in skin texture less obvious than for cooked frozen peas.

For the 1939 delay series, peas of the Tall Alderman variety were again used. Immediately after vining, one lot of the peas was frozen and

the others were hermetically sealed in tin cans and immersed in cooled water, the temperatures being 1.7, 7.2, 23.9, and 37.8° C. (35, 45, 75, and 100° F.) for different lots. At the end of three-, six-, and nine-hour delay periods, cans of each were opened, the tenderometer readings were obtained, and the peas were frozen in the usual manner. The purpose of packaging in tin during the delay periods was to minimize the effect of desiccation. It was thought that desiccation might be an important factor in the toughening of skins during delay. At the end of delay periods the peas were frozen and after five months of storage, penetration measurements were made; the results are summarized (Tables 6 and 7).

TABLE 6

Effect of Delay on Skin Texture of Frozen Peas, 1939

Delay period	Temperature during delay	Tenderometer reading or field run	Calculated composite	Penetration values				
				Size 7	Size 6	Size 5	Size 4	Size 3
hr.	*°C.*	*lb./sq. in.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
0	.....	95.6	1.18	1.47	1.22	1.05	0.75	.....
3	1.7	100.4	1.26	1.56	1.31	1.09	0.88	0.63
3	7.2	98.6	1.27	1.70	1.33	1.06	0.80	0.62
3	23.9	101.4	1.48	1.95	1.58	1.17	1.00	0.73
3	37.8	100.4	1.76	2.14	1.92	1.57	1.15	0.86
6	1.7	100.8	1.44	1.83	1.58	1.16	0.93	0.74
6	7.2	101.6	1.30	1.61	1.38	1.15	0.90	0.63
6	23.9	101.8	1.73	2.05	1.76	1.37	1.06	0.87
6	37.8	103.2	1.98	2.23	2.19	1.82	1.53	0.98
9	1.7	102.4	1.37	1.82	1.45	1.13	1.02	0.70
9	7.2	102.0	1.31	1.71	1.38	1.10	0.85	0.66
9	23.9	102.6	1.40	1.74	1.44	1.22	1.02	0.87
9	37.8	107.2	2.13	2.27	2.27	2.15	1.72	1.10

TASTERS' JUDGMENT OF QUALITY RELATED TO TEXTURE VALUES

Grade A—1.15 kg. or less
Grade B—Not exceeding 1.35 kg.
Grade C—1.42 kg. or more

The results show that even when the peas were sealed in tin cans during delay periods in order to protect them from desiccation, the penetration values of the skins increased. After three hours of delay at each of the temperatures, the penetration values of the skins of all sizes of peas, except Sizes 4 and 5 held at 7.2°C.(45°F.), increased significantly. The penetration values of most of the sizes of peas increased significantly between the third and sixth hours of holding at each of the temperatures used. The penetration values of fewer of the sieve sizes changed significantly between the sixth and ninth hours than at the earlier stages of delay, and of those which did change the nine-hour sample was usually more tender than the six-hour sample except in the case of the sample held at 37.8°C.(100°F.). Thus, skins of most of the peas toughened during the first three hours and again between the third and sixth hours at all of the holding temperatures. There was less tendency for the skins to

change between the sixth and ninth hours, but of those which did change the six-hour sample usually was tougher.

A comparison of the skin texture of peas held for the *same period* but at different temperatures (Table 7) is also of interest. For the most part, skin texture of peas held at 1.7°C.(35°F.) for three, six, or nine hours did not differ from those held for the same periods at 7.2°C.(45°F.); and of those which did differ, the skins of the peas held at the higher temperature were usually more tender. The skins of almost all of the sizes of peas held at 7.2°C.(45°F.) for three, six, or nine hours were more

TABLE 7

Significance of Difference of Mean Penetration Values of Table 6

Items compared				Level of t values for different sieve sizes		
1[1]		2				
Period of delay	Temperature of delay	Period of delay	Temperature of delay	t value exceeds 1% level	t value exceeds 5% level but less than 1% level	t value less than 5% level
hr.	*°C.*	*hr.*	*°C.*	*Size*	*Size*	*Size*
3	1.7	0	.....	6, 5, 4	7	.....
6	1.7	3	1.7	7, 6, 5, 3	.....	4
6	1.7	9	1.7	6, 3	4	7, 5
3	7.2	0	.....	7, 6	.....	5, 4
6	7.2	3	7.2	.....	5, 4	—7, 6
6	7.2	9	7.2	.....	.....	—7, 6, 5, 4, —3
3	23.9	0	.....	7, 6, 5, 4	.....	.....
6	23.9	3	23.9	6, 5, 3	.....	7, 4
6	23.9	9	23.9	7, 6, 5	.....	4, 3
3	37.8	0	.....	7, 6, 5, 4	.....	.....
6	37.8	3	37.8	6, 5, 4, 3	.....	7
9	37.8	6	37.8	5, 3	.....	7, 6, 4
3	7.2	3	1.7	7	—4	6, —5, —3
3	23.9	3	7.2	7, 6, 4	5, 3	.....
3	37.8	3	23.9	7, 6, 5, 4	.....	.....
6	1.7	6	7.2	7, 6, 3	.....	5, 4
6	23.9	6	7.2	7, 6, 5, 4, 3	.....	.....
6	37.8	6	23.9	7, 6, 5, 4	3	.....
9	1.7	9	7.2	.....	.....	7, 6, 5, 4, 3
9	23.9	9	7.2	5, 4, 3	.....	7, 6
9	37.8	9	23.9	7, 6, 5, 4, 3	.....	.....

[1] Column 1 has the higher value unless the sieve size in the analysis column is preceded by a minus sign.

tender than those held at 23.9°C.(75°F.) for the same periods, the sizes six and seven for the nine-hour period being the only exception. The skins of almost all sizes of peas held at 23.9°C.(75°F.) for three, six, or nine hours were more tender than those held for the same periods at 37.8°C. (100°F.), Size 3 for the three-hour delay period being the only exception. Thus, there was little difference in skin texture owing to holding peas at 1.7°C.(35°F.) or at 7.2°C.(45°F.). However, as the temperature of holding them was increased to 23.9°C.(75°F.) and to 37.8°C.(100°F.), the skins became less tender.

Since the texture of peas with a given tenderometer reading identifies the quality of peas for many persons, it is of interest to correlate the degree of toughening of skins resulting from delay with the tenderometer

readings. The conditions of growing, freezing, and testing the peas (reported in Tables 2 and 6) were the same and, therefore, such a comparison can be made with these samples. The peas reported in Table 6, which were frozen without delay after vining, had a tenderometer reading of 95.6. Their skins had approximately the same penetration values as the same variety of peas reported in Table 2, which had a tenderometer reading of 101.8. Delay samples shown in Table 6, which had approximately the same skin texture values as peas reported in Table 2 having a tenderometer reading of 118, were peas delayed for six hours at 1.7°C.(35°F.), three or six hours at 23.9°C.(75°F.), or three, six, or nine hours at 37.8°C. (100°F.). The results indicate that delay periods at these temperatures result in toughening of skins equal to that which occurs when the tenderometer reading increases approximately 16 pounds at this stage of maturity.

SUMMARY

The resistance of skins of cooked peas to penetration and of cotyledons to crushing was used as a measure of the texture of cooked peas. Higher penetration and crushing values denote tougher texture.

1. The variations in texture of fresh peas of different sieve sizes were less than the variations for one sieve size taken from pods which appeared to be of different maturity, even when the variety, field, and day of harvest were the same. Therefore, sieve sizing was considered an unsatisfactory means of separating peas of a run-of-the-field sample into texture groups.

2. The longer the peas were left in the field the greater was the penetration value of a given size of cooked frozen peas. Harvesting at a later date and discarding the large sizes of peas, would, therefore, be an undesirable practice. The penetration values for peas harvested on different dates were related to the tenderometer readings of the samples.

3. When skins of frozen peas which were boiled for five minutes were tough, boiling them for 15 minutes usually did not correct this condition.

4. Vining toughened both the skins and cotyledons of frozen peas.

5. Delay between vining and freezing toughened the skins but not the cotyledons of frozen peas. This was true for hand-shelled peas as well as for viner-shelled peas. Most of the toughening of skins of vined peas was found to occur during the first four to six hours of delay and there was little or no additional toughening thereafter up to eight or nine hours.

6. Washing vined peas in water to remove the vine juice before delay periods did not prevent toughening of skins, but less toughening occurred than when the peas were held without washing.

7. Cooling the peas, even to 1.7°C.(35°F.), during delay periods did not prevent toughening of their skins within three hours but less toughening occurred at lower temperatures. Delay of six hours at 1.7°C.(35°F.), three or six hours at 23.9°C.(75°F.) or three, six, or nine hours at 37.8°C. (100°F.) toughened skins of frozen peas as much as an increase in maturity reflected by an increase from 102 to 118 in tenderometer reading.

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PROTECTION OF ASCORBIC ACID DURING ITS EXTRACTION FROM PLANT TISSUES

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Comparatively satisfactory methods used by Fujita and Iwatake (1935), Musulin and King (1936), Bessey (1938), and others have been developed for the extraction of ascorbic acid from plant tissues using metaphosphoric acid as a protective agent, but there is need of further study in the adaptation of the methods to different types of tissues. The time and duration of the critical stage in the extraction process in tissues of high oxidative activity have not been fully investigated.

The present studies are concerned with some of these problems. The tests have been restricted to a study of methods of preservation of the ascorbic acid present in the tissues at the beginning of the tests, such as by cooking and utilization of an adequate volume and concentration of metaphosphoric acid. Traces of dehydroascorbic acid which may have formed previously in some of the material were not determined.

EXPERIMENTAL PROCEDURE

Immature seeds of soybeans were chosen for the initial studies because preliminary tests had shown that very rapid oxidation of their vitamin C would occur during extraction unless protective conditions were maintained. Later tests were also conducted with carrot, potato, banana, cowpeas, spinach, and kale.

Selection of samples of soybeans for the tests were made on the basis of uniformity with respect to size and degree of maturity, hence the values obtained do not necessarily indicate the ascorbic acid content of the "average run" of the seeds. Uniform samples were desirable in order to make comparative tests of different methods of extraction. The rapidity of changes in size and in total ascorbic acid content of the seeds from day to day imposed distinct limitations upon the number of tests which could be made under identical conditions.

Four to six-gram samples of the immature seeds were rapidly ground (for 45 seconds in some tests and 60 seconds in others) with acid-digested sand in a measured quantity of metaphosphoric acid—eight per cent acetic acid—extracting solution, the concentration of the metaphosphoric acid varying in different tests.[1] The extract was strained through several layers of cheesecloth and the residue rinsed with five c.c. of the acid mixture. The residue was then reground for 30 seconds with another volume of the acid mixture equal to that used in the first grinding and rinsed as before. The conditions for the third grinding were similar to

[1] Later work has shown in agreement with results reported by Bessey (1938) that the acetic acid has little or no influence upon the results.

those of the second. This was followed by a fourth and final rinsing with 10 c.c. of the acid.

Extracts which passed through a filter readily and whose residues washed easily were mixed with filtercel and filtered; others which were slow to filter and whose residues were more difficult to wash were centrifuged. The soybean extracts were filtered because the extracts could be recovered more rapidly by this procedure and time was an important factor in some of the tests. This procedure is satisfactory, however, only for small samples. The cooked material was more difficult to filter than the uncooked. The use of larger quantities of filtercel in the former was found to be helpful.

The extract was made up to a 100-c.c. volume with additional acid of the same concentration as was used in the extraction, and aliquots adapted to the vitamin C content of the tissue were used in titrating against a standardized solution of purified (ether-extracted) 2, 6 dichlorophenolindophenol.

Assays were made on both cooked and uncooked samples of most of the types of tissues employed in the tests. Immediately after weighing the uncooked sample it was immersed in the desired volume of the metaphosphoric acid extracting solution. Samples to be cooked were placed in 30-c.c. beakers and 10 c.c. of boiling water were added. They were then covered with watch glasses and placed in a large covered beaker containing boiling water. The cooking period in minutes for the different types of material were as follows: soybeans and cowpeas, 25; carrot and potato, 15; banana and kale, 5; spinach, 3. After being cooked the samples were cooled rapidly in an ice-water bath and the acid was then added.

RESULTS

The values[2] shown (Table 1) were obtained in tests with soybeans of the Rokuson variety, one of the vegetable types now coming into use in this country. Each sample contained 10 seeds and weighed approxi-

TABLE 1

Effect of Cooking and Quantity of Metaphosphoric Acid Used in Extraction of Soybeans (Rokuson Variety) on Yield of Ascorbic Acid[1]

Acid solution	3 c.c.		6 c.c.		9 c.c.	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
2% HPO_3 + 8% CH_3COOH	.090	.191	.177	.186	.190	.184
5% HPO_3 + 8% CH_3COOH	.158	.186	.194	.189	.199	.191
8% HPO_3 + 8% CH_3COOH	.176	.189	.195	.196	.204	.193

[1] Given in milligrams per seed; ten seeds per sample; approximate weight 5.75 grams.

mately 5.75 grams. The effect of variations in volume and concentration of metaphosphoric acid employed in grinding the tissues on the results of vitamin C determinations is to be observed. The preservative effect of cooking is also apparent. Three c.c. of the two-per cent metaphosphoric

[2] The validity of the ascorbic acid values herein reported depends upon the supposition that the reducing value of the plant-tissue extracts under the conditions employed is due entirely to ascorbic acid.

acid solution afforded full protection in extracting the ascorbic acid from the cooked soybeans, whereas only the nine-c.c. volume was adequate with the uncooked sample. The latter volume and concentration of acid fully protected the vitamin in the raw soybeans, whereas three c.c. of the eight-per cent metaphosphoric acid containing a larger total quantity of the acid did not entirely prevent oxidative action, supposedly because of incomplete coverage.

Investigations with Rokusun soybeans were also undertaken to determine the quantity of ascorbic acid removed at each grinding when different concentrations and different volumes of metaphosphoric acid were used. The quantity of acid used in rinsing the residues after each grinding was so adjusted that the total volume of acid used in grinding and washing was in each instance the same. All were adjusted to make a total of 14 c.c. in each of the three grindings and washings. The extract and washings from the first grinding were made up to 50 c.c. in volume with additional acid of the same concentration as was used in extraction, and a 10-c.c. aliquot was used for titration. The extracts from the second and third grindings were also each made up separately to 50 c.c. in volume and appropriate aliquots were titrated.

Seeds were harvested several days later from the same plants. There had been no rain during this period and these seeds had a somewhat lower vitamin C content. Ten seeds were used in each test. The results (Table 2) are due entirely to differences in conditions during grinding. By equalizing the total quantity of acid used in each extraction, variations in different tests owing to differences in volumes of acid passing through a given quantity of pulverized tissue tended to be eliminated. The preservation of the ascorbic acid was found to be directly dependent upon the absolute quantity of metaphosphoric acid used in maceration, particularly in the first grinding. Above the minimum quantity that is necessary for adequate coverage during maceration, differences in volume of extracting solution employed in grinding were not significant provided a sufficiently high concentration of metaphosphoric acid was used. Differences in pH of the medium were doubtless somewhat involved in yielding the results in the early phases of grinding, but such variations were of very short duration, as the first extracts were all made up rapidly to a 50-c.c. volume with additional acid. The speed of the oxidative reaction is indicated by the changes which occurred during the 45-second period of the first grinding when the vitamin was not adequately protected. Because of the small quantity of ascorbic acid extracted in the final rinse, the end point was difficult to read. The values for this determination are consequently not as accurate as the others. A somewhat similar series of tests was conducted with six-gram samples of potato tissue. The ratio of the total quantity of vitamin C removed in the first extract to the probable amount originally present varies somewhat less with the use of different volumes and concentrations of metaphosphoric acid than in soybeans.

The metaphosphoric acid requirements for the protection of vitamin C during its extraction from six-gram samples of fresh and cooked tissues of several different types selected for uniformity as to age and general condition were investigated. Marked differences in the vitamin C values

obtained by using different volumes of the two-per cent metaphosphoric extracting solution are to be noted in the results shown (Table 3). The requirement is relatively low for the two leafy vegetables, but high for the two legumes. Two to three c.c. of the extracting solution, a quantity equivalent to one-third to one-half c.c. per gram of tissue, appear to pre-

TABLE 2

Ascorbic Acid Removed From Soybeans (Rokuson Variety) at Each Extraction Using Different Concentrations and Different Volumes of Metaphosphoric Acid in Eight Per Cent Acetic Acid[1]

Acid solution	3 c.c.	6 c.c.	9 c.c.
2% HPO_3 + 8% CH_3COOH			
First extraction 45 sec.	0.044	0.072	0.090
Second extraction 30 sec.	0.027	0.036	0.044
Third extraction 30 sec.	0.012	0.013	0.014
Fourth extraction rinse only	0.004	0.003	0.002
Total	0.087	0.124	0.150
Percentage of total amount found, removed in first extraction	50.6	58.1	60.0
Percentage of probable original amount removed in first extraction	28.4	46.5	58.1
5% HPO_3 + 8% CH_3COOH			
First extraction 45 sec.	0.060	0.088	0.091
Second extraction 30 sec.	0.032	0.047	0.047
Third extraction 30 sec.	0.013	0.014	0.015
Fourth extraction rinse only	0.006	0.003	0.003
Total	0.111	0.152	0.156
Percentage of total amount found, removed in first extraction	54.0	57.9	58.3
Percentage of probable original amount removed in first extraction	38.7	56.8	58.7
8% HPO_3 + 8% CH_3COOH			
First extraction 45 sec.	0.077	0.091	0.092
Second extraction 30 sec.	0.048	0.046	0.045
Third extraction 30 sec.	0.017	0.018	0.018
Fourth extraction rinse only	0.004	0.005	0.003
Total	0.146	0.160	0.158
Percentage of total amount found, removed in first extraction	52.7	56.9	56.9
Percentage of probable original amount removed in first extraction	49.7	58.7	59.3

[1] Results given in milligrams per seed; ten seeds per sample.

vent oxidation of the vitamin C in spinach and kale, whereas soybeans and cowpeas require more than twice that quantity. In fact, such a large volume of the two-per cent metaphosphoric acid in proportion to the size of the sample is required that maceration is difficult except by the use of a comparatively large quantity of sand. A smaller volume of extracting solution having a higher concentration of metaphosphoric acid was found to be preferable. Banana and potato yielded normal values when extracted with one c.c. per gram of tissue, and carrots with one-half c.c.

Cooking, under the conditions of rapid heating employed, had a preservative effect on the vitamin C in bananas, carrots, cowpeas, and soy-

TABLE 3

Reducing Values[1] Obtained From Six-Gram Samples Using Different Volumes of Extracting Solution With Each Grinding

Product	5 per cent metaphosphoric acid		2 per cent metaphosphoric acid + 8 per cent acetic acid									
	6 c.c.		9 c.c.		6 c.c.		3 c.c.		2 c.c.		1 c.c.	
	Fresh	Cooked	Fresh	Cooked	Fresh	Cooked	Fresh	Cooked	Fresh	Cooked	Fresh	Cooked
Carrot	5.58	5.48	5.83	5.71	5.48	5.68	5.44	5.54	5.04	5.27	3.65	4.85
Green Mountain potato	18.3	.....	17.8	.....	17.7	.....	17.0	.....	14.9	.....	10.3	.....
Banana	10.6	10.5	11.6	10.7	10.6	10.6	7.6	10.8	4.2	9.9	1.6	9.7
Groit variety[2] cowpeas	39.7	39.8	36.5	39.6	30.8	38.6	12.9	37.8	.....	.....	.....	.....
Nanda soybeans	34.4	34.2	33.0	.....	30.5	35.6	11.7	34.3	.....	.....	.....	.....
Spinach	78.8	70.7	69.5	62.1	79.4	72.3	81.0	69.8	71.4	69.5	70.0	64.7
Kale	216.0	201.0	224.0	203.0	220.0	185.0	221.0	236.0	235.0	232.0	206.0	221.0

[1] Calculated as milligrams of ascorbic acid per 100 grams of tissue. [2] Young stage—before development of extensive purple mottling in seed coat.

beans. It caused a loss in spinach. The values obtained with kale were somewhat irregular and were doubtless caused in part by variations in actual content of ascorbic acid in different samples, possibly as a result of partial shading of the leaves during their growth—Murphy (1939) and Reid (1938, 1942). In none of the vegetables tested did the vitamin C values in cooked tissues exceed those of fresh tissues when determinations of the latter were made under conditions preventing oxidation. The lower values found in cooked carrots and bananas extracted with one c.c. and to a smaller degree in those extracted with two c.c. are probably a consequence of unfavorable pH relations.

DISCUSSION

The extremely rapid oxidation of ascorbic acid which occurs during maceration of fresh tissues of high oxidative activity, such as in immature soybeans and cowpeas, makes imperative the provision of protective conditions from the moment the first crushing of cells begins.

It is not the final concentration of metaphosphoric acid in the extracting solution that is of primary importance but the concentration in the early phases of grinding. A practical procedure for the routine extraction of tissues of high oxidative activity would be the use of a fairly high concentration (five to eight per cent) of metaphosphoric acid solution for the early stages of extraction and of a two-per cent concentration for the later phases, such as the final washing of the residue and making of solutions up to volume. Bessey (1938) found that complete extraction and adequate protection can be obtained with three per cent of metaphosphoric acid. No tests were made with this concentration of metaphosphoric acid in the present investigations.

The difficulties encountered in the selection of approximately uniform samples for the tests herein described have indicated that the selection of representative samples of tissues for assaying their vitamin C content constitutes a problem equal in importance to the choosing of a method for its extraction. Each general type of material imposes its own problems. A method of selection of samples from bulky material, such as spinach and potatoes, should be chosen only after a thorough investigation of the quantitative distribution of the vitamin in different regions and in different ages of tissues and determination of the relative quantity of these tissues in relation to the whole.

It should be noted also that the present results indicate that immature soybeans and cowpeas have a vitamin C content equal to that of fresh garden peas. Two of the vegetable types of soybeans, Rokuson and Nanda, at the edible stage yielded values of 32 to 34 milligrams per 100 grams. Moreover, the vitamin C is well preserved during cooking if the beans are heated very rapidly.

SUMMARY

The preservation of vitamin C during its extraction with metaphosphoric acid from tissues of high oxidative activity depends (1) upon the amount of the acid used in the early phases of grinding and (2) upon sufficient volume to furnish coverage of all parts of the sample at the moment the first crushing of cells begins.

Spinach and kale were found to have relatively low; soybeans and cowpeas high; and carrot, banana, and potato intermediate requirements of metaphosphoric acid.

Cooking had a preservative effect on vitamin C in tissues of high oxidative activity. Similar values were obtained in cooked and in fresh samples but the latter had considerably higher metaphosphoric acid requirements.

The results indicate that immature soybeans and cowpeas have a vitamin C content comparable to that of fresh garden peas. Two of the vegetable types of soybeans, Rokusum and Nanda, at the edible stage yielded values of 32 to 34 milligrams per 100 grams. The vitamin C is well preserved in cooking if the beans are heated very rapidly.

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SOME CHEMICAL CHANGES ACCOMPANYING TENDERIZATION OF BEEF

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The development of the Tenderay process for tenderizing beef introduced a new set of storage conditions, and led to the present study of the chemical changes that might be characteristic of the new process. The literature, from which only a few references are cited here, concerning changes that occur in meat during storage provided ample evidence that the course and extent of such complex reactions would vary appreciably with both temperature and time—Hoagland, McBride, and Powick (1917); King (1941); Musulin and King (1936); Needham (1932); Rentschler and Nagy (1940); Moran and Smith (1929); Newton *et al.* (1937); and Winton and Winton (1937).

EXPERIMENTAL PROCEDURE

Tenderness was measured by a specially devised penetrometer. Juiciness, flavor, and additional tests of tenderness were measured by the usual organoleptic procedure. The bactericidal effects of the ultraviolet lamps used in the process have been considered in earlier publications by Oppenheimer and Stern (1939).

The changes selected for quantitative study were (a) the increase in sulfhydryl groups, primarily as a measure of incipient changes in protein —a change that is especially involved in denaturization; (b) the increase in soluble nitrogen as a measure of proteolysis and related types of hydrolysis; (c) the decrease in vitamin C (ascorbic acid), primarily as a very sensitive measure of oxidative changes; and (d) the increase in serum concentration of hemoglobin-type materials, measured by the Evelyn photoelectric colorimeter when equipped with filter No. 540 M (used for hemoglobin determinations).

The initial determinations were made on muscle tissue, mostly the club and sirloin steak portions, of the short loin of carcasses 24 hours after being slaughtered. These were carefully checked by the inspectors in the plant as being typical products. Pairs of short loins from the same animal were utilized, one serving as the control in the conventional chill cooler at 1.67°C.(35°F.), the other being treated by the Tenderay process. The treatment consisted of holding them in the process room for 48 hours at 15.6°C.(60°F.) with a relative humidity of 85 to 90 per cent in the presence of ultraviolet lamps. Corresponding cuts from both loins were used in the determinations and duplicate runs were made on each determination.

Soluble nitrogen was measured by the A.O.A.C. method (Kjeldahl-Gunning-Arnold) (1935). Sulfhydryl groups were determined by titration with .01 N iodine in acid solution; the values are expressed as

milliliters of .01 N iodine per 100 grams of tissue. The titrations were corrected for ascorbic acid values.

The photoelectric colorimeter (Evelyn type) was used to follow changes in the color density of the press juice as measured with filter No. 540 M. The press juice (.2 ml.) was first diluted with distilled water (four ml.) to give a suitable concentration range.

The method of Bessey and King (1933) as modified by Musulin and King (1936) was followed in the estimation of vitamin C. Dehydroascorbic acid was not determined because of the extremely minute amount of the substance present and because of the unsatisfactory nature of the method that was used by King (1941). The dye was standardized daily against $Na_2S_2O_3$.

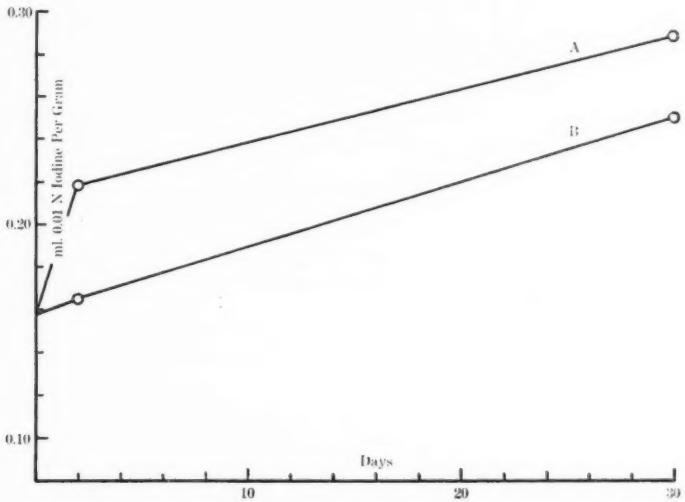

FIG. 1. Titration of sulfhydryl groups in beef during storage for tenderization. Curve A, beef held at 15.6°C.(60°F.) for 48 hours; Curve B, the opposite side of beef, held for control tests under standard cold-room conditions.

Typical changes in sulfhydryl content are shown (Fig. 1); it is evident from the curves that there is a relatively rapid increase in sulfhydryl groups during the Tenderay processing, compared with the more gradual increase that is characteristic of the low-temperature controls.

The results from the soluble nitrogen analyses (Fig. 2) show a distinct increase in the processed meat during the first 48 hours. The average increase during this period was 17 per cent for the processed meat, compared with 4.3 per cent for the low-temperature controls. The corresponding increases during a 30-day period, corresponding to common trade practice for low-temperature storage, were 32 and 20 per cent, respectively.

The vitamin C changes were nearly parallel during both types of treatment. There was a steady decrease in ascorbic acid titration values during storage, but the accelerated tenderization did not cause a marked

increase in the rate of vitamin loss (Fig. 3). Each point on the curves given in the figures represents an average of at least 12 determinations.

Changes in serum concentration of the hemoglobin-type compounds are shown (Fig. 4).

DISCUSSION

The rise in titratable sulfhydryl content during the early period of processing is characteristic of the early stages of protein denaturization, Schmidt (1939), and may be considered as a sensitive measure of certain protein changes that are in part concurrent with tenderization. The parallel rise in soluble nitrogen is also in part a measure of protein alteration that accompanies the process of tenderization—both changes being sensitive to the use of an elevated temperature. The changes in sulyhydryl

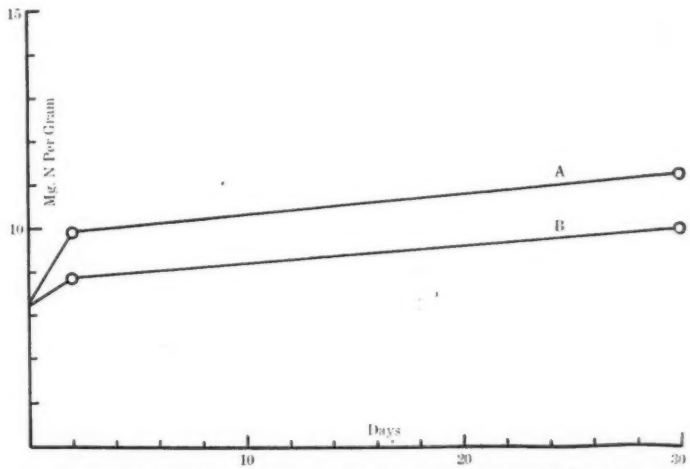

FIG. 2. Changes in soluble nitrogen in beef held under standard cold-room conditions (Curve B) and under conditions used in the Tenderay process (Curve A).

values are, in general, more sensitive than changes of a hydrolytic nature. Protein reactions of the denaturization type are known not to follow the simple principle that governs many reaction rates, namely, an approximate doubling of the reaction rate per 10 degrees rise in temperature. The protein denaturization tendency may be extremely sensitive to a critical temperature range (Schmidt, p. 407); the reaction rate may increase by 600 times during a 10-degree temperature rise instead of doubling or tripling. Beyond the 48-hour period, the rates of change were practically identical in the two series of samples.

The antiscorbutic value of fresh meat has been emphasized by many explorers, notably Stefansson, who has observed primitive peoples to be completely protected from evident vitamin C deficiency through long periods, and even cured from scurvy, with no other source of the vitamin than that provided by fresh meat, *either* cooked or raw. The greater retention of ascorbic acid in the Tenderay processed meat is also of interest

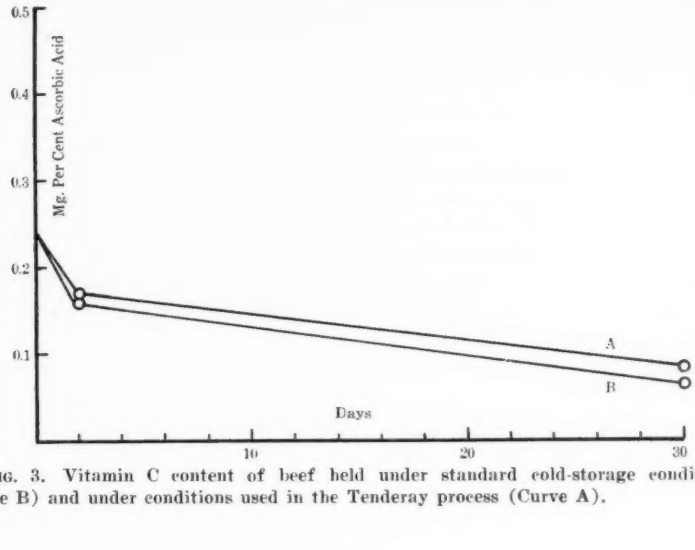

FIG. 3. Vitamin C content of beef held under standard cold-storage conditions (Curve B) and under conditions used in the Tenderay process (Curve A).

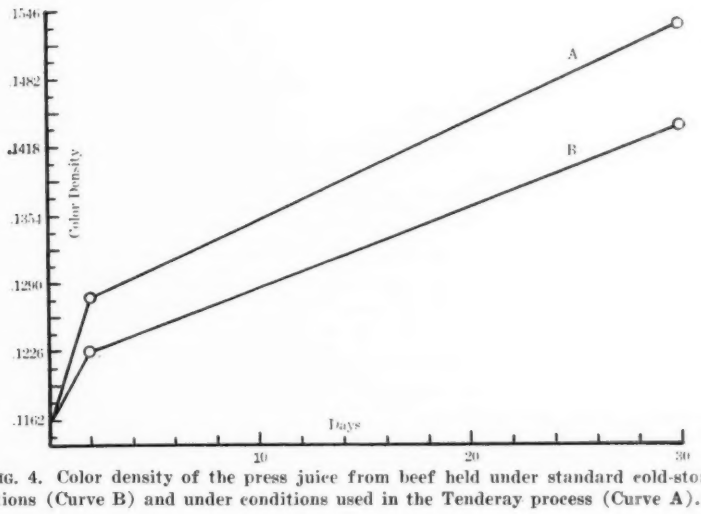

FIG. 4. Color density of the press juice from beef held under standard cold-storage conditions (Curve B) and under conditions used in the Tenderay process (Curve A).

in relation to undesirable oxidative changes in addition to the minor significance that it may have from a nutritional point of view. At marketing time the Tenderay meat studied in the present investigation retained 69 per cent of its original antiscorbutic value compared with 37 per cent in the meat held according to standard cold-room practice. Although the vitamin C content of lean beef is very low, other forms of meat are known to have higher antiscorbutic values, and the use of the process is not being confined to beef. The quick rise in sulfhydryl groups probably serves both to increase proteolytic activity and to inhibit oxidation of ascorbic acid, Oppenheimer and Stern (1939). We have viewed the present data concerning ascorbic acid primarily as an index of oxidative change, but in view of Stefansson's observation (1936) that American meat supplies can provide complete protection from scurvy, the vitamin C factor is one of nutritional interest.

SUMMARY

Among the chemical changes that characterize the tenderization of beef by the Tenderay process (approximately 48 hours at 60°F.), compared with standard low-temperature tenderization (approximately 30 days at much lower temperature), are (a) a more rapid rise in sulfhydryl content; (b) a more rapid increase in soluble nitrogen compounds; (c) a comparable rate of disappearance of vitamin C (higher value at time of consumption); and (d) a more rapid rise in hematin-type pigments in the press juice.

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EFFECT OF VARIOUS COOKING METHODS UPON SUBJECTIVE QUALITIES AND NUTRITIVE VALUES OF VEGETABLES

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The claims of manufacturers of nationally advertised cooking utensils concerning the saving of minerals and vitamins cooked in their utensils, as well as the superior color, flavor, texture, and odor of the product obtained, present a problem to the thoughtful consumer. Since there is little experimental evidence to aid in resolving this problem, it seemed that an investigation into mineral and vitamin retention in vegetables cooked in various utensils, as well as into the more elusive qualities just mentioned, might prove of value.

EXPERIMENTAL PROCEDURE

The cooking utensils chosen for the experimental work were those representative of various cooking methods: an enamelware open kettle in which vegetables were boiled in a comparatively large quantity of water; two types of aluminum "waterless" cookers, whose heavy construction and tightly fitting covers facilitate the cooking of vegetables in their own juices or in a very small added quantity of water; and a small aluminum pressure saucepan in which vegetables were cooked in steam at 15 pounds pressure. The latter is a comparative newcomer among cooking utensils and is distinguished from the usual pressure cooker by the rapidity with which steam is generated and the ingenious simplicity of construction which makes for ease in using.

Vegetables used for the tests on color, flavor, texture, and odor may be grouped as follows: First are those containing carotenoids as their chief pigment, which previous experimental work by Halliday and Noble (1933) has shown to be remarkably stable to varying degrees of heat and to changes in the hydrogen-ion concentration of the cooking solution. Carrots were selected as representative of this group. Second are those whose chief pigment is chlorophyll, the destruction of which McMillan (1929) has shown is favored by high temperatures and becomes rapid when the hydrogen-ion concentration of the cooking solution shifts to the acid side of neutral. Green beans, peas, asparagus, and spinach represent this group. Third are those vegetables containing sulfur glycosides, a component which gives members of the cabbage family their tangy, pleasing flavor when raw but which is hydrolyzed under certain cooking conditions to yield disagreeable flavors and odors, as shown by Simpson and Halliday (1928). Representative of this group are cabbage and broccoli, likewise pigmented with chlorophyll, and cauliflower and white turnips, chiefly pigmented with flavones, which have been shown to be colorless in acid and yellow in alkaline solution. The vegetables for these tests and likewise those for the chemical analyses were bought on the

Chicago retail market, and all were fresh and in good condition when used.

Vegetables cooked in the open kettle according to directions in "Hows and Whys of Cooking" by Halliday and Noble (1933) were arbitrarily used as the standard for comparison, while for waterless and pressure-saucepan cooking of vegetables, directions found in the advertising material accompanying the cookers were followed explicitly. Four serving portions of vegetables for each method of cooking were prepared as recommended, with an occasional change in the way the vegetable was cut, so that no especial advantage might be considered to have accrued to the standard owing to the method of preparation. Cooking was synchronized so that one lot of the various vegetables was ready for judging at the same time, and other conditions were controlled so as to eliminate variables as much as possible, as described in detail by Brinkman (1941). For scoring, the vegetable which served as the control was so labelled, while those cooked by other methods were not known by the three judges, who were familiar with good standards for cooked vegetables. A simple score was used as follows:

+6 greatly superior to the standard
+4 superior to the standard
+2 slightly superior to the standard
= equal to the standard
—2 slightly inferior to the standard
—4 inferior to the standard
—6 greatly inferior to the standard.

Averages of the scores are given (Table 1).

The vegetables used for comparing the retention of calcium, phosphorus, and vitamin C by the different methods of cooking were carrots, green beans, peas, cabbage, cauliflower, and white turnips. One series of analyses was carried out on each of these vegetables using triplicate samples in the raw state and cooked by the various methods. Samples for analyses were weighed from each of the four serving portions and placed in very thin cheesecloth bags, a device recommended by Noble that made it easy to calculate back to the raw weight. These samples, plus any remaining vegetable needed to make up a four-serving portion, were then cooked. The liquid remaining after cooking was discarded, since it was decided that it is seldom served with the vegetable or, if served, rarely eaten. For the mineral determinations, the contents of the bags were dry ashed, and aliquots of the ash solutions so obtained were analyzed for calcium in duplicate by the Shohl and Pedley (1922) modification of the McCrudden (1911-1912) method and for phosphorus by the Fiske and Subbarow (1925) method. Ascorbic acid was extracted from the raw and cooked vegetable samples with an eight-per cent trichloroacetic acid solution containing two per cent metaphosphoric acid, using the general procedures of Bessey and King (1933), the dye being standardized according to the method of Menaker and Guerrant (1938). Aliquots of each extraction were titrated in triplicate; the results are given (Table 2).

TABLE 1

Average Scores of Vegetables Cooked in Various Utensils as Compared With Those Cooked in Open Kettle[1]

Vegetable	Method of preparation	Color			Flavor			Texture			Odor		
		Waterless cookers		Pressure saucepan	Waterless cookers		Pressure saucepan	Waterless cookers		Pressure saucepan	Waterless cookers		Pressure saucepan
		1	2		1	2		1	2		1	2	
Carrots	⅛-in. slices	=	.....	—1	+1	.....	+1	=	.....	—1	.....	.....	.....
Beans, green, 1	2 or 3 pieces	—5	.....	—1	—3	.....	—1	—1	.....	—1	.....	.....	.....
Beans, green, 2	Narrow strips	—1	.....	—3	+1	.....	—1	—1	.....	=	.....	.....	.....
Peas, 1	.....	—4	.....	+2	=	.....	+1	—1	.....	=	.....	.....	.....
Peas, 2	.....	.....	—2	+3	.....	=	=	.....	—1	+1	.....	.....	.....
Asparagus	Tips and butts	—6	.....	—2	—3	.....	—4	=	.....	=	.....	.....	.....
Spinach	Leaves	—3	.....	=	—4	.....	—1	—3	.....	=	—3	.....	=
Cabbage, green	Shredded	—5	.....	—4	—5	.....	—5	—1	.....	—1	—5	.....	—4
Broccoli	Flowerlets	—5	.....	—4	—7	.....	—5	—7	.....	—6	—2	.....	—1
Cauliflower	Flowerlets	—3	.....	—3	—3	.....	=	—5	.....	—3	—1	.....	=
Turnips, white	¾-in. cubes	.....	—2	—1	.....	—5	+2	.....	—1	—1	.....	—3	=

[1] All utensils were made of aluminum except an enamel saucepan used for the open-kettle method in cooking carrots, green beans, and cabbage.

TABLE 2

Retention of Calcium, Phosphorus, and Vitamin C in Vegetables Cooked in Various Utensils[1]

Vegetable	Method of preparation	Calcium				Phosphorus				Vitamin C			
		Open kettle	Waterless cookers		Pressure saucepan	Open kettle	Waterless cookers		Pressure saucepan	Open kettle	Waterless cookers		Pressure saucepan
			1	2			1	2			1	2	
Carrots	2 or 3 slices in	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
	length, 1 across	94	90	.....	89	84	81	.....	83	50	50	.....	45
Beans, green	2 or 3 pieces	121	100	.....	99	87	83	.....	81	51	49	.....	40
Peas	———	105	.....	94	96	.....	.....	.....	.....	46[2]	.....	48	58
Cabbage, green	Shredded	98	97	.....	100	60	68	.....	92	40	61	.....	53
Cauliflower	Flowerlets	92	.....	100	94	73	.....	78	85	49	.....	49	64
Turnips, white	¾-in. cubes	95	.....	100	87	.....	.....	.....	.....	46	.....	66	70

[1] All utensils were made of aluminum except an enamel saucepan used for the open-kettle method in cooking carrots, green beans, and cabbage. [2] This figure is an average of values obtained on two samples, while all others are averages of three separate samples of one lot of vegetables.

DISCUSSION

From the results of the subjective scoring of the vegetables for color, flavor, texture, and odor (Table 1) it is evident that no general conclusion can be drawn that would apply to all vegetables cooked in any one type of utensil.

The non-brassicaceous vegetables were for the most part about the same when cooked in the pressure saucepan as when cooked in the open kettle. Two exceptions were asparagus, which was inferior in color and flavor, and peas, which were superior in color.

The brassicaceous vegetables showed considerable variation. White turnips were entirely satisfactory, cauliflower was satisfactory with respect to flavor and odor, while cabbage and broccoli were definitely inferior when cooked in the pressure saucepan.

With the exception of carrots and beans that were shredded, all the vegetables were considered inferior in most respects when cooked in the waterless cookers. As might be expected, this inferiority was most marked in the case of the brassicaceous vegetables. Reference to the table will reveal deviations from these general statements.

A consideration of the nutritive values retained in various methods of cooking indicates that retention of calcium in all methods for all vegetables ranged from 87 to 121 per cent, with the open-kettle method highest in three out of six cases, the waterless cooker highest in two cases, and all three methods nearly equal in the sixth case. The actual gain in calcium by beans and peas is in line with the findings of Noble and Halliday (1937), which shows that when vegetables are cooked in water containing as much as 30 parts per million of calcium, as does Chicago city water, they either gain calcium or show smaller losses than when distilled water is used. The retention of phosphorus in four vegetables ranged from 60 to 87 per cent when cooked in the open kettle, from 68 to 83 per cent when cooked in the waterless cookers, and from 81 to 92 per cent when cooked in pressure saucepan. Although the range for the open-kettle method is definitely lower than that for the pressure saucepan, beans showed the highest retention by the former method, while carrots were about equal by the two methods.

The retention of reduced ascorbic acid in six vegetables ranged from 40 to 51 per cent for the open-kettle method, from 48 to 66 per cent for the waterless cookers, and from 40 to 70 per cent for the pressure saucepan. In the case of green beans and carrots the vitamin C retention was about equal by open-kettle and waterless-cooker methods, both being somewhat higher than by pressure-saucepan cooking.[1] Cabbage, which was finely shredded, showed best retention when cooked in the waterless cooker and

[1] Realizing the desirability of having more conclusive evidence on each vegetable, this laboratory now has in progress a more extensive study on ascorbic acid retentions including three to four repetitions of all cooking procedures. From among the above listed vegetables, green beans have again been studied and the new results show percentage retentions by the pressure saucepan method from twenty to thirty-five higher, whereas by the open kettle method the results are in the same range as here reported.

least when cooked in the open kettle. For the remaining three vegetables those cooked in the pressure saucepan retained the vitamin best, while the other two methods gave results appreciably lower with cauliflower and peas, and turnips were appreciably lower only by the open-kettle method. Since vitamin C is soluble in water, it might seem that vegetables cooked in the open kettle, with its large volume of water, would show much greater losses of this nutrient than vegetables cooked by methods using little or no water. Gould, Tressler, and King (1936) have noted, however, that the greatest loss of vitamin C occurs from the time the vegetable is plunged into the boiling water to that time when the boiling point is again reached. That this is due to the activity of enzymes present in raw vegetables which react to oxidize ascorbic acid whenever a cut surface is exposed to the air has been shown by Mack (1936). Thus two factors, solubility of vitamin C and the rapidity of its destruction by oxidizing enzymes, both influenced by a third variable, the method of cutting the vegetables, are operating simultaneously in unknown degrees. The combined effects of these variables may account for the fact that the losses of vitamin C are not much more and sometimes even less in vegetables cooked in large volumes of water than in those cooked in small volumes.

SUMMARY

In general it may be concluded, therefore, that so far as subjective qualities are concerned, the small pressure saucepan is as satisfactory as the open kettle for the cooking of all green vegetables except asparagus, as well as for the cooking of carrots and white turnips. However, the pressure saucepan seems less desirable for other brassicaceous vegetables, namely, cabbage, broccoli, and cauliflower. On the other hand, the waterless cookers produce a completely satisfactory product only with carrots and green beans cut in narrow strips. From the standpoint of nutritive values, no one of the cooking methods shows definite superiority over another so far as calcium is concerned, when the cooking water contains calcium, nor is the loss great in any case. With phosphorus and vitamin C, on the other hand, the variation in retention is greater, on the whole; and the pressure saucepan has, on the average, a slight advantage over the other methods.

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VITAMIN B COMPLEX CONTENT OF BEERS, ALES, AND MALT TONICS

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Until recently the belief prevailed that fermented malt beverages, such as beers and ales, contain very little, if any, vitamins. Most of the vitamin tests made by workers in the past were restricted to the determination of vitamin B_1. Scheunert and Schieblich (1935) reported the first quantitative results on a dark beer. They found three international units of B_1 and about 7.4 biological units of B_2 per 100 milliliters of beer. A year later Donovan and Hanke (1936) found values ranging from 2.5 to five I. U. of B_1 per 100 ml. in five commercial samples of beer. From their tests they estimated the vitamin G or vitamin B_2 content of these five commercial beers to be at least twice that of the vitamin B_1 content or at least 15 to 30 micrograms per 100 ml. Laufer, Brenner, and Laufer (1940) last year determined the vitamin B_1 content of 32 samples of beers and ales and of four malt tonics. They found values ranging from 1.9 to 4.8 I. U. per 100 ml. for the beers, the average being 3.25. For the ale samples, the values varied from .9 to 4.7, the average being 2.96 I. U. per 100 ml. Variations from 10 to 23, with an average of 16.6 I. U. per 100 ml., were obtained for the malt tonics. Recently, Levine (1941) reported that whereas most of the thiamin or vitamin B_1 present in the wort is absorbed by the yeast during fermentation (which would account for the low vitamin B_1 content of beers and ales), the vitamin B_2 or riboflavin remains in the beer. Using fluorometric and animal-assay procedures, he was able to establish that the riboflavin content of beer is from 38 to 47 micrograms per 100 ml. or, on the average, 150 micrograms per 12-ounce bottle (approximately 350 ml.).

During recent years many new important vitamins of the B complex have been discovered and found to exist in considerable quantities in brewers' yeast by Bandier and Hald (1939); Hennessy and Cerecedo (1939); Kodicek (1940); Schumacher and Heuser (1940); Arnold, Schreffler, and Lipsius (1941); Schwarz, Laufer, Laufer, and Brenner (1941); and Strong, Feeney, and Earle (1941). In addition, new accurate and rapid methods for the assay of these vitamins have been worked out. These new developments suggested to the authors the importance of testing malt beverages for the presence of several components of the vitamin B complex.

METHODS OF ASSAY

The following important vitamins of the B complex group were determined in nine samples of beer, ale, and malt tonics: thiamin (B_1), riboflavin (B_2), nicotinic acid, and pantothenic acid.

For thiamin determination the sulfite cleavage modification by Schultz, Atkin, Frey, and Williams (1941) of the fermentation method of Schultz,

Atkin, and Frey (1937, 1938) was employed. Riboflavin was assayed by the microbiological method of Snell and Strong (1939) using *Lactobacillus casei-ε*. Similarly, pantothenic acid was determined using the same organism, employing the procedure of Pennington, Snell, and Williams (1940). Finally, nicotinic acid was assayed by the microbiological procedure of Snell and Wright (1941) in which the organism *Lactobacillus arabinosus 17-5* is used.

DISCUSSION OF RESULTS

The vitamin B_1 values obtained are similar to, but somewhat less than, those previously reported by Laufer, Brenner, and Laufer (1940). The values reported (Table 1) are probably more accurate, because the sulfite-

TABLE 1

Vitamin B Complex Content of Beers, Ales, and Malt Tonics

Samples	Vitamin B_1		Vitamin B_2	Nicotinic acid	Pantothenic acid
	Micrograms per 100 ml.	I. U. per 100 ml.	Micrograms per 100 ml.	Mg. per 100 ml.	Micrograms per 100 ml.
Beer 1	2.67	0.89	25.3	0.783	120
Beer 2	4.02	1.34	22.7	0.868	85
Beer 3	1.47	0.49	33.0	0.801	100
Ale 1	8.19	2.73	30.4	0.694	90
Ale 2	1.11	0.37	38.0	0.891	100
Ale 3	2.88	0.96	32.0	0.866	93
Malt tonic 1	7.14	2.38	38.7	1.040	100
Malt tonic 2	0.00	0.00	57.3	1.790	133
Malt tonic 3	48.60	16.20	49.3	1.650	130

splitting procedure has improved the fermentation method by eliminating the effect of substances other than vitamin B_1. With the exception of malt tonics, the vitamin B_1 content of malt beverages, such as beers and ales, is low. However, contrary to former beliefs, the remaining vitamins of the B complex, for which assays were made, are present in appreciable quantities in fermented malt beverages. The riboflavin, nicotinic acid, and pantothenic acid contents appear to be about the same in beers and ales, and somewhat less than the amounts found in malt tonics. The average riboflavin content of beers, ales, and malt tonics is 27, 33.5, and 48.4 micrograms per 100 ml., respectively. Similarly, the nicotinic acid averages are .817, .817, and 1.49 mg. per 100 ml., and the pantothenic acid average is 102, 94, and 121 micrograms per 100 ml. of beer, ale, and malt tonics, respectively. Although no claims can be made for beer as providing the daily requirements of some components of vitamin B complex, if three 12-ounce bottles of beer (about one liter or slightly more than one quart) were consumed daily, an appreciable percentage of the daily requirements of these components would be supplied, a fact hitherto not recognized. Based on the above figures, one liter of beer contains about 300 micrograms of vitamin B_2 or about 10 per cent of the daily minimum requirements. Similarly, one liter of beer contains about 8.2 mg. of nicotinic acid or, roughly, 44 per cent of the daily minimum requirement. The minimum daily requirement of pantothenic acid is not known at present.

SUMMARY

Owing to the fact that tests of malt beverages were usually confined to a determination of vitamin B_1, the belief has prevailed that these products are devoid of vitamins. In the present tests three different brands of beers, ales, and malt tonics were assayed by microbiological methods for thiamin, riboflavin, nicotinic acid, and pantothenic acid contents. With the exception of malt tonics, the low thiamin content of fermented malt beverages was confirmed. This probably is due to absorption of thiamin by the yeast in the course of fermentation. However, contrary to former beliefs, the remaining vitamins of the B complex, for which assays were made, were found in appreciable quantities in fermented malt beverages. The following average values were found for beer, ale, and malt tonics, respectively: riboflavin, 27, 33.5, 48.4 micrograms per 100 ml.; nicotinic acid, .817, .817, 1.49 mg. per 100 ml.; pantothenic acid, 102, 94, 121 micrograms per 100 ml.

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A STUDY OF ORGANISMS FOUND IN LACTIC ACID FERMENTATION OF LETTUCE

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The organisms discussed here were isolated from natural and inoculated lactic acid fermentations of shredded, salted lettuce. The technology and chemistry of the process and of the resulting product have been reported by Hohl and Cruess (1940). In this paper the flora of these fermentations is analyzed.

EXPERIMENTAL PROCEDURE

Two natural fermentations (*A* and *B*) of shredded and salted lettuce differed from each other only in the source of the lettuce, *A* being Salinas Valley lettuce and *B* being Imperial Valley lettuce. Two other fermentations (*C* and *D*), using Imperial Valley lettuce, were respectively inoculated with active cultures of the heterofermentative *Lactobacillus brevis* and the homofermentative *L. plantarum*, both of which had been isolated from Fermentation *A*.

The total count of viable organisms was made in tomato juice tryptone agar at intervals during all the fermentations by the usual dilution plate method. Each time counts were made about 10 colonies were picked from the dilution most suitable for transfer. Half of these were surface colonies and half were subsurface colonies and all the types of organisms represented were included at each picking.

These organisms were inoculated into a liver infusion medium, McClung and McCoy (1934); purified by streaking on sterile plates; and classified according to Bergey *et al.* (1939), Hucker and Pederson (1930), Orla-Jensen (1919), and Pederson (1936 and 1938).

RESULTS AND DISCUSSION

The original bacterial flora was mainly of the gram-negative, catalase-positive type, but within the first day or two the gram-positive, catalase-negative, lactic-acid bacteria appeared and continued to be the most prevalent bacteria throughout the remainder of the fermentations (Tables 1 and 2). In Fermentation *A* (Table 1) *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* and *L. brevis* (Orla-Jensen) Bergey *et al.* were present simultaneously throughout the fermentation, with the former soon taking the dominant role. In Fermentation *B* (Table 2) *L. brevis* was much more abundant than *L. plantarum*. During the early stages of this fermentation several cultures of *Leuconostoc mesenterioides* (Cienkowski) Van Tieghem [1] were isolated. After the sixth day these had all disappeared.

[1] These cultures were classifiable as *Leuconostoc mesenterioides* on the basis of all characteristics except slime formation from sucrose.

TABLE 1

Organisms Found in Fermentation A (Uninoculated Salinas Valley Lettuce)

Time	Number of colonies picked	Number of gram-negative, catalase-positive bacteria	Number of yeasts	Number of gram-positive, catalase-negative bacteria				
				Lactobacillus brevis	*L. plantarum*	*L. buchneri*	*Leuconostoc mesenteroides*	Others
days								
0	10	10	....	....	....	....	....	.....
1	8	7	....	....	1	....	....	.....
2	10	6	....	3	1	....	....	.....
3	8	....	....	2	6	....	....	.....
4	8	1	....	1	3	....	....	1 lost 2 blank[1]
5	9	....	....	....	7	....	....	2 blank
6	10	....	4	3	2	....	....	1 lost
7	10	....	3	....	6	....	....	1 blank
8	10	....	....	5	3	....	....	2 blank
9	10	....	1	1	8	....	....	.....
11	10	....	....	3	7	....	....	.....
14	10	....	....	3	4	....	....	3 lost
Total	113	24	8	21	48	....	....	12

[1] Blank cultures were those which failed to grow on the initial liver culture.

TABLE 2

Organisms Found in Fermentation B (Uninoculated Imperial Valley Lettuce)

Time	Number of colonies picked	Number of gram-negative, catalase-positive bacteria	Number of yeasts	Number of gram-positive, catalase-negative bacteria				
				Lactobacillus brevis	*L. plantarum*	*L. buchneri*	*Leuconostoc mesenteroides*	Others
days								
0	5	3	....	2	....	....	....	.....
1	9	6	1	....	....	....	....	2 lost
2	10	....	....	2	....	....	1	4 lost 3 blank[1]
3	10	0	....	3	....	....	7	.....
4	10	....	....	6	....	....	2	2 blank
5	10	....	....	6	....	1	2	1 blank
6	10	....	....	4	1	2	1	2 blank
7	10	....	1	5	1	2	....	1 blank
8	12	....	3	3	5	....	....	1 lost
Total	86	9	5	31	7	5	13	16

[1] Blank cultures were those which failed to grow on the initial liver culture.

TABLE 3

Organisms Found in Fermentation C (Imperial Valley Lettuce Inoculated With Lactobacillus brevis)

Time	Number of colonies picked	Number of gram-negative, catalase-positive bacteria	Number of yeasts	Number of gram-positive, catalase-negative bacteria				
				Lactobacillus brevis	*L. plantarum*	*L. buchneri*	*Leuconostoc mesenteroides*	Others
days								
0	10	....	....	4	....	....	....	6 blank[1]
1	10	....	1	9	....	....	....	.....
2	10	....	....	7	....	2	....	1 blank
3	10	....	2	8	....	....	....	.....
4	10	....	1	8	1	....	....	.....
5	10	....	....	4	5	....	....	1 blank
6	12	....	1	2	9	....	....	.....
8	10	....	2	3	5	....	....	.....
10	10	....	2	2	2	....	....	4 blank
12	11	....	9	1	1	....	....	.....
Total	103	....	18	48	23	2	....	12 blank

[1] Blank cultures were those which failed to grow on the initial liver culture.

TABLE 4

Organisms Found in Fermentation D (Imperial Valley Lettuce Inoculated With Lactobacillus plantarum)

Time	Number of colonies picked	Number of gram-negative, catalase-positive bacteria	Number of yeasts	Number of gram-positive, catalase-negative bacteria				
				Lactobacillus brevis	*L. plantarum*	*L. buchneri*	*Leuconostoc mesenteroides*	Others
days								
0	10	....	....	....	5	....	....	5 blank[1]
1	10	....	....	....	9	....	....	1 blank
2	10	....	....	2	7	1	....	.....
3	10	....	....	....	9	....	....	1 blank
4	10	....	1	7	1	....	....	1 blank
5	10	....	1	....	7	....	....	2 blank
6	10	....	2	....	7	....	....	1 blank
8	10	....	1	....	9	....	....	.....
10	11	....	4	....	4	....	....	2 blank 1 lost
12	12	....	5	1	6	....	....	.....
Total	103	....	14	10	64	1	....	13 blank 1 lost

[1] Blank cultures were those which failed to grow on the initial liver culture.

The inoculated fermentations, *C* and *D*, (Tables 3 and 4) showed that inoculation with a small amount of pure culture was effective in controlling the dominant flora of the fermentation.

Lactobacillus plantarum maintained its dominance throughout Fermentation *D*, became an appreciable factor in the later stages of Fermentation *C*, and was also dominant in Fermentation *A*. Fermentation *B* was stopped a little earlier than the others, but during its last few days *L. plantarum* began to be important here also. This organism seems to be the most suitable for these lettuce fermentations. *Lactobacillus buchneri* (Henneberg) Bergey *et al.* was found occasionally in all except Fermentation *A*.

In the later stages of all the fermentations, there occurred film-forming yeasts which did not sporulate on vegetable wedges or gypsum blocks. They attacked the lactic acid which was being produced by the bacteria.

SUMMARY

The most prevalent organisms found in lactic acid fermentations of lettuce were *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.*, and *L. brevis* (Orla-Jensen) Bergey *et al.*, and some nonsporulating, film-forming yeasts. Occasional cultures of *L. buchneri* (Henneberg) Bergey *et al.* and *Leuconostoc mesenterioides* (Cienkowski) Van Tieghem were also isolated.

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TRACE ELEMENTS IN FOODS[1]

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This topic can be discussed from the standpoint of the value of these elements in nutrition, from the analytical angle, from the toxicological viewpoint, or it can be discussed with reference to all of these. For adequate discussion of any one of the above phases of the subject, more ample space would be required. It is our purpose to mention briefly in this discussion all of the phases of the topic referred to above. When the problem has been discussed in the past, some authors have stated that one of the difficulties in deciding the subject matter to be dealt with under the title of this paper is to answer the question "When does an element become a 'trace' element?" Then they have proceeded to fail to answer their question and confined their attention to certain elements which are undoubtedly present in foodstuffs only in small amounts and yet play some important role. Others have referred to the fact that the spectrographic method has established that certain chemical elements occurring in foods in mere traces—quantities so small that they had been completely overlooked—were extremely important. They have then mentioned that some animals and plants suffer disastrous results to health and life if these elements are not present, and then proceeded with a discussion of some of these.

It seems best to confine this discussion to those elements for which there is quantitative knowledge of their presence in plant and animal tissues and to those for which quantitative knowledge is lacking but whose toxicology has been investigated. Therefore, we shall attempt to define what is meant by (1) "trace" element; (2) nutritive trace element; (3) nonnutritive, nontoxic trace element; and (4) nonnutritive, toxic trace element. A nutritive trace element may also be toxic but this group is so small that it has not been subdivided. It is proposed that trace element be defined as any element which occurs quite widely distributed yet rarely exceeds 20 milligrams per kilogram (20 parts per million or 20 micrograms per gram) in foodstuffs *as consumed.* As an illustration, fluorine would certainly be considered by everyone a trace element since it commonly occurs in foodstuffs in quantities less than 20 p.p.m., but a few natural foods are known in which it occasionally exceeds this quantity. Aluminum and copper may also be used as similar illustrations.

A nutritive trace element is defined as one which has definitely been shown to play a role in normal biological processes of man or the higher forms of animal life.

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A nonnutritive, nontoxic trace element is defined as one which for higher forms of life is not nutritive and which has not been shown to cause some form of deleterious effect in higher forms of animal life when it is incorporated in the diet in quantities not exceeding 100 p.p.m.

A nonnutritive, toxic trace element will then be defined as one which is not nutritive but is known to cause some form of deleterious effect in man or higher animals when administered in quantities of 100 p.p.m. or less in the diet.

NUTRITIVE TRACE ELEMENTS

COBALT

There is wide distribution of cobalt in foods with considerable variation in content, and it has been found in most organs of the body. Analyses of foods by Ahmad and McCollum (1939) of a large number of dried materials have shown that the content ranges from .18 to .47 p.p.m., beans and corn containing, respectively, .14 and .42 p.p.m., whereas cereals range from .06 to .08 p.p.m. Cobalt is known to be required in the amount of about one mg. per day by certain animals, particularly by sheep and cattle, to prevent enzootic marasmus characterized by loss of appetite, emaciation, and anemia; but there is no conclusive evidence for its need in human nutrition. According to Godden (1939) cobalt is less toxic than nickel, but when administered to dogs, for example, shows deleterious effects when the quantity exceeds 30 mg. per kg. of animal per day.

COPPER

Copper occurs in all foods and tissues that have been examined but in widely varying amounts. Of a number of fresh vegetables that have been analyzed by Levine, Culp, and Anderson (1932) the copper content varies from .021 p.p.m. for broccoli, to 1.38 p.p.m. for turnip greens, whereas that of the fresh muscle of a large number of fish was found by Parks and Rose (1933) to vary from 1.4 p.p.m. for the common sunfish to 4.1 for the ling. Oysters range in value from 28 to 93 p.p.m. according to Levine, Remington, and Culp (1931). Foods of animal origin have been shown by Kogan and Nasyrova (1938) to range from approximately .4 p.p.m. for egg white to 22 p.p.m. for beef liver. Compilation by Beeson (1941) of the analyses of 169 foodstuffs by various authors gave an average value for copper on a dry-weight basis of 12 p.p.m. Kehoe, Cholak, and Story (1940a) have shown that the mean blood value for 60 normal individuals was 1.14 p.p.m., which is in general agreement with other investigators. Values for human liver vary from 3 to 9.1 p.p.m. and bone may contain as much as 11.9 p.p.m. Eggleton (1940a) reported that 13 organs and tissues of 26 humans ranged from 6.4 p.p.m. for muscle to 40.2 p.p.m. for liver. Lamb and Lloyd (1939) have stated that the copper content of tomato products may be as high as 50 p.p.m., while the tolerance for the United Kingdom is 100 p.p.m.

Hart and his associates at the University of Wisconsin were the first to show that a nutritional anemia, characterized by low hemoglobin content and low erythrocite count of the blood, developed when animals were placed on diets low in copper. They further showed that levels of copper

as low as .05 mg. per day produced the optimum response in rats if the daily intake of iron was adequate. This work has been confirmed and extended by these same investigators and by others. Copper is therefore essential for the normal nutrition of many of the higher forms of animals and probably also for man; for bibliography see Elvehjem (1935). Daniels and Wright (1934) feel that the amount of copper in the ordinary diet does not greatly exceed the requirements and they suggest that the diet of preschool children should not include less than a quantity which will furnish .1 mg. of copper per kg. of body weight per day. It should be pointed out that although the evidence is somewhat controversial, there is little room for doubt, according to Elvehjem (1935), that copper is required by man.

The chronic toxicity of copper, in my opinion, has been incompletely investigated. However, it has been shown by Hopkirk (1938-39), for example, that when 100 c.c. of one-per cent copper sulfate was administered daily for 33 to 77 days to a group of sheep, death resulted and that the liver concentration of these animals varied from 1,950 to 4,030 p.p.m. on a dry-weight basis. Because of the emetic action of copper acute toxicity studies can be made only on those animals which do not vomit, and for this reason accurate determinations of the acute oral toxic doses for rats, mice, and guinea pigs only have been established by Woodard and Calvery (1941). The LD 50 doses using copper chloride are for rats, mice, and guinea pigs, 140, 190, and 31 mg. per kg., respectively. An extensive investigation of the toxicity of copper was made by Mallory and Parker (1931) who have concluded that acute poisoning with copper causes anemia, hemoglobinurea, necrosis of hepatic and renal cells, and pigmentation. Mallory and his associates think that copper causes chronic effects on the liver especially when associated with alcoholic liquors. Rats fed 525 p.p.m. for 12 months, according to Coulson, Remington, and Lynch (1934), showed no evidence of intoxication from the metal. The source of copper was "green" oysters and copper sulfate. Before a final conclusion can be reached as to the amounts and conditions under which copper causes chronic toxicity, further investigations will be necessary.

IRON

Waller (1935) summarized the analyses of 235 items used as human foods, and the iron content averaged 19.52 p.p.m.; 113 of these items had over 10 p.p.m. and 15 of them, over 50 p.p.m., while 20 had 2.5 p.p.m. or less. Iron has long been known to be an essential substance in nutrition, and often because of the comparative magnitude of its requirement and the amount present, it is not considered a trace element, but under our definition it will be included. No toxicity has been demonstrated for iron. Because of almost universal knowledge of iron and its nutritional significance and the excellent textbook discussions, our comments in this presentation are being limited.

IODINE

Iodine is present in small quantities in nearly all foods and in all the tissues of man and animals, with the highest concentration being present in the thyroid, which in man contains about 75 per cent of the

total iodine of the body. Compilation of analyses by Beeson (1941) of 50 items of foodstuffs gave an average value of .465 p.p.m. on the dry-weight basis or between .1 and .3 p.p.m. on the basis of the condition of the foods as they are consumed. It is essential for normal nutrition and when the daily intake is less than .002 mg. (two micrograms) per kg. body weight, simple goiter may occur; but when two micrograms per kg., or approximately 100 micrograms per day, are taken in with the daily diet, there is adequate amount for normal iodine metabolism. Deficiencies occur not only in man but in many domestic animals. Iodine is probably not required per se but is essential for the synthesis of thyroglobulin, the hormone of the thyroid gland, the presence of adequate amounts of which, according to Lerman (1941), is required for the normal function of all cells of the body.

Although iodine is absolutely essential for nutrition, it is an excellent example of an element which can cause toxicity when ingested in small quantities over a period of time. The phenomenon is called iodism and takes the form of various rashes and of coryza, headache, conjunctivitis, laryngitis, bronchitis, etc. Less common are stomatitis, parotitis, anorexia, etc.

MANGANESE

Data collected by Beeson (1941) show that the average manganese content of 166 items of food analyzed by different individuals was 48 p.p.m. on a dry-weight basis, or between 10 and 20 p.p.m. on a fresh-weight basis, which seems rather high in the light of investigations by Kehoe, Cholak, and Story (1940b). In a single investigation the mean daily intake of a normal adult American over a period of 28 days was 4.28 mg. of manganese, of which there is practically no storage. The findings of Kehoe, Cholak, and Story (1940a,b) and of Bruckman and Zondek (1939) have shown that fresh tissue contains .2 to .6 p.p.m., with human blood having an average content of .15 p.p.m. When the diet of the rat is free or very low in manganese content, there is disturbance of the female estrus cycle and sterility of the male with failure of normal growth and congenital debility in the young. Although there is no conclusive evidence that manganese is required by man, it has been claimed by Daniel and Everson (1934) that children should get .2 to .3 mg. of it per kg. of their diet.

The chronic toxicity of manganese when it is included in the diet has not been carefully investigated, but its toxicity as reported by Flinn, Neal, Reinhart, Dallavall, Fulton, and Dooley (1940) for man is demonstrated by the fact that nine men exposed to 30 mg. per cubic meter of air showed muscular in-co-ordination with difficulty in walking as the first sign of deleterious effects. Similar effects have been obtained by others on rabbits. For a review of certain phases of the literature concerning this element see von Oettingen (1935).

ZINC

Beeson (1940) reported analyses of 72 foodstuffs by different investigators for their zinc content. These showed an average of 32.5 p.p.m. on a dry-weight basis, which would mean approximately 10 p.p.m. on a

fresh-weight basis. Analyses of 13 human organs and tissues ranged in zinc content from 43 (cerebrum) to 245 (liver) p.p.m., and the zinc content of blood of Europeans averaged 6.75 p.p.m. and of Chinese, 6.79 p.p.m., according to Eggleton (1940a,b).

Zinc has been shown by Hubbel and Mendel (1927) and by Todd, Elvehjem, and Hart (1934) to be beneficial to rats and mice when added to diets which are very low in zinc. An interesting occurrence of zinc is its presence in insulin even after the insulin has been several times recrystallized, and more recently it has been found by Scott and Fisher (1935, 1938) that the mean zinc content of the pancreas of 18 diabetics was only one-half that of 14 normals. No instance in zinc deficiency in man or animals under natural conditions has yet been reported.

Sutton (1939) showed that oral administration of .1 to .5 per cent of the diet as zinc sulfate resulted in anemia and death in 10 to 12 weeks at the .5-per cent level, whereas the .1-per cent level showed very little toxicity. Pharmacologic action of soluble zinc salts resemble those of copper but are milder. They are sometimes used as emetics and locally as astringents and antiseptics. No systemic toxic effects have been observed in man after oral administration. The LD 50 doses of zinc chloride for rats, mice, and guinea pigs are, respectively, 350, 350, and 200 mg. per kg. as shown by Woodard and Calvery (1941).

NONNUTRITIVE, NONTOXIC TRACE ELEMENTS

ALUMINUM

The best estimate that can be given for the aluminum content of foodstuffs is a value somewhere between 10 and 30 p.p.m. The daily intake has been estimated by Burn (1932) and by Monier-Williams (1935) as about 13 mg.; see also Kehoe and associates (1940 a,b) and Underwood (1940) for further reviews of the literature. The controversy concerning the aluminum in human tissue is probably due to difficulty with methods of determination, but more recent investigations indicate that aluminum is regularly present in small amounts. The amount of aluminum absorbed from the gastrointestinal tract is very small and there is no convincing evidence that it is required in the metabolism of man or animals. Studies on the toxicity of aluminum indicate that harmful amounts are not absorbed from the gastrointestinal tract, and the administration of 200 mg. per day to man has caused no untoward effect. However, it has been shown by Cox, Dodds, Wigman, and Murphy (1931) that aluminum can cause a disturbance of phosphorus metabolism in guinea pigs when the animals are maintained on a diet to which aluminum is added, probably owing to a combination of the aluminum with the phosphorus in the gastrointestinal tract.

BERYLLIUM

If beryllium is present in living tissue, methods are not adequate to determine it, and there is no known evidence for its necessity in nutrition. It is well known, however, as shown by Guyatt, Kay, and Branion (1933), that beryllium rickets can be produced by its addition to the diet of ani-

mals, but usually these diets are low in calcium and in all instances the beryllium concentration has exceeded 100 p.p.m.

BORON

Boron is widely distributed in plant tissues where it has been shown to play a significant role in their normal metabolism, but such a role has not been demonstrated for the animal kingdom. Analyses of 37 items of foodstuff have shown, according to Beeson (1940), that the average content of fresh materials is at least 10 p.p.m. There is no conclusive proof that when included in the diet of normal animals or man in quantities of 100 mg. per kg. or less it causes any deleterious effects. However, according to Sollmann (1936) its effects on the digestive system and the kidneys indicate that it would be undesirable even at very low levels in the diets of individuals with digestive or renal disturbances.

CHROMIUM

There are so few analyses of food and tissue for their content of chromium that it seems unwise to try to present an average figure, and there is no evidence that chromium is necessary for the normal nutrition of man or animals. Salts of chromium with a valence of three present a striking contrast in their toxicity to salts of chromium with a valence of six, the latter being very toxic whereas the former have a low toxicity, according to Atkatsuka and Fairhall (1934). The toxicity owing to the chromic and chromate forms of chromium is probably attributable primarily to their powerful oxidizing properties. They have local ulcerating effects on the skin and when ingested the gastrointestinal tract is intensely irritated. The chromate ion is also toxic systemically when absorbed or injected. When chromium is fed to the rat in the form of its chromous salts at a level of 100 p.p.m. in the diet it produces no deleterious effects, according to Conn, Webster, and Jensen (1932).

SILICON

Because of the ubiquitous distribution of silicon in the soil surface, it is to be assumed silicon can be found in all plant and animal tissues, and analyses by King and Belt (1938) show that this is undoubtedly the case. Analysis of fetal tissue shows a normal range from 40 to 400 p.p.m. and for adult tissue, from 100 to 2,000 p.p.m. on a dry-weight basis, the values for lungs being highest and those for muscle lowest in adult tissue, whereas the reverse is true for fetal tissue. Blood of man and animals on the average contains 10 p.p.m. There is no evidence to indicate that silicon is required in the nutrition of man or animals, and there is no evidence of its oral toxicity. However, it should be pointed out that numerous cases of silicosis, a pulmonary disease, have been reported owing to the inhalation of dusts containing silicon.

STRONTIUM

Strontium occurs to some extent in plant tissues and has been reported occasionally in traces in soft tissues of man and in human bone of different ages. Although it may play a role in the nutrition of some of the forms of animal life, its function in the higher forms has not been demon-

strated. Its toxicity when administered in the diet at low levels has not been demonstrated but when included in quantities sufficient to furnish one gram per kilogram of body weight per day, it produces severe rickets in young rabbits. Woodard and Calvery (1941) found that the LD 50 doses of strontium chloride for rats and mice are, respectively, 2,250 and 3,100 mg. per kg.

TIN

Analyses of foodstuffs and tissues for tin have not been very extensive, but those that have been made indicate that the content is not greater than 10 p.p.m. on a fresh-weight basis and the average less than one p.p.m. The highest concentrations of tin in animal tissues appear in the glandular organs, but no biological role has been attributed to it. No toxicity has been demonstrated when tin was included in the diet in quantities of 100 p.p.m. or less, but a few times this amount of soluble tin, when administered orally, may cause gastric and intestinal irritation to such an extent that nausea, vomiting, and diarrhea result, but no systemic toxicity has been demonstrated. Woodard and Calvery (1941) have shown that the LD 50 doses of stannous chloride ($SnCl_2$) for the rat and mouse, two species which do not vomit, are, respectively, 700 and 1,200 mg. per kg.

TITANIUM

Beeson (1940) reported that analyses of 43 items of food have shown an average titanium content of 4.8 p.p.m. on a dry-weight basis. So far there has been no demonstration of the role of titanium in nutrition. Neither has its chronic toxicity been shown when incorporated in the diet in small quantities; for example, titanium salts when fed in quantities exceeding 100 p.p.m. to sheep, dogs, and other animals for periods of two months or longer have shown no deleterious effect, according to Askew (1931) and Lehmann and Herget (1927).

NICKEL

Analyses of 22 foodstuffs by various authors show an average of .95 p.p.m. on a dry-weight basis, according to Beeson (1940). Even though older analyses indicate that nickel is more widely distributed in animal tissues than is cobalt, there are no recent data on the nickel content of biological materials, and there are no animal experiments to demonstrate its significance in nutrition. Although it is more toxic than cobalt, showing some toxicity in dogs when administered in single doses of approximately 20 mg. per kg. of animal, there are no data available which show that it causes chronic toxic effects in quantities less than 100 mg. per kg. of diet.

NONNUTRITIVE, TOXIC TRACE ELEMENTS

SILVER

Analyses of food and feces indicate that the daily intake of silver is about .06 to .08 mg., and 10 per cent of the tissues analyzed have shown the presence of silver in quantities usually less than one p.p.m., according to Kehoe, Cholak, and Story (1940a,b). The nutritive significance of silver has not been established. Although it is quite generally realized

that solutions of salts of silver are germicidal and bactericidal, their principal manifestation of toxicity when ingested in small amounts over long periods of time for higher forms of life, particularly for man, is a discoloration of the skin and tissues referred to as argyria. An excellent discussion of this problem has been presented by Hill and Pillsbury (1939).

BISMUTH

Because of the small amounts of bismuth in the soil, it is to be expected that if found in plant and animal tissues the quantities would be small. As a matter of fact, according to Sheldon and Ramage (1931), spectrographic examination of human tissues has not revealed the presence of bismuth. More recent examination by Rusoff and Gaddum (1938) of normal newborn rats likewise did not reveal the presence of bismuth. It follows as a corollary to the above that the biological significance of bismuth in normal nutrition has not been studied, and the only biological significance considered is its usefulness in therapeutics. No adequate feeding experiments have been performed to establish its chronic toxicity in animals. When it is used in therapeutics, the quantities rarely exceed the administration of more than 100 mg. of soluble bismuth compounds three times weekly. Sollmann (1936) states that if such administration is continued over a period of time, the person may exhibit conditions which are the result of its systemic action, such as pigmentation of the gum margins, stomatitis, jaundice, nephritis, dermatitis, and occasionally other miscellaneous types of reactions.

CADMIUM

Like bismuth, cadmium is present only in traces in soils and therefore would not be expected to be present in appreciable quantities in plant products. Its presence in human tissues and rat tissues which have been examined has not yet been revealed, according to Sheldon and Ramage (1931) and Rusoff and Gaddum (1938). There is no information concerning its nutritive significance, and the only interest in cadmium from a biological viewpoint is that it can readily cause both acute and chronic toxicity when present in small quantities in the form of its soluble salts.

The toxicology of cadmium is most interesting because of the acute symptoms which result so soon after the ingestion of very small amounts. The history of cadmium poisoning has been discussed by Prodan (1932). More recently five outbreaks of cadmium poisoning have been reported by Frant and Kleeman (1941). In these outbreaks at least 50 persons were involved. The smallest amount of cadmium which produced noticeable effects was in materials containing 15 p.p.m. In the other four outbreaks, the cadmium content of the materials ingested was, respectively, 67, 160, 300, and 530 p.p.m. The different types of material consumed were flavored frozen ices, lemonade, punch, and gelatin dessert. The quantities consumed were evidently small since it would not be expected that a person would consume large quantities of frozen ices. In another instance which has been reported to the author by Osburn (1941), 175 men showed symptoms of cadmium poisoning after drinking lemonade which had been stored in cadmium-plated metal containers for only about one and one-half hours. The lemonade contained 100 p.p.m. of cadmium, and

when left in the containers for 24 hours, it was found to contain 250 p.p.m. The onset of the symptoms often occurs within 10 minutes after the ingestion of the material. The syndrome appears as violent acute gastritis with nausea, cramps, vomiting, diarrhea, weakness, and is often accompanied by metallic taste. Chronic toxicity studies on rats by Prodan (1932), Wilson, DeEds, and Cox (1941), and Fitzhugh and Meiller (1941); and on dogs by Calvery, Grant, and Montgomery (1941) have demonstrated that deleterious effects are shown when animals ingest as low as 15 p.p.m. in their diet. The LD 50 doses of cadmium chloride for rats, mice, and guinea pigs are, respectively, 87, 175, and 63 mg. per kg. as shown by Woodard and Calvery (1941). Chronic toxicity in man, according to Legge (1924), has occurred only where cadmium is used in the industry. At this point its seems advisable to issue an urgent word of warning against the use of cadmium-plated food containers of any kind; this is especially important in the case of foods containing acids.

FLUORINE

The average fluorine content, according to McClure (1939), of a large number of foods which have been analyzed is approximately .8 p.p.m. with a few natural foods running relatively high in their fluorine content. The daily excretion of fluorine by normal individuals is from one to three milligrams. In addition to the fluorine naturally present in foods, it is sometimes added in the form of cryolite sprays to protect certain foods against insect infestation. It is an element which probably should be included among the nutritive trace elements since evidence is accumulating that in quantities less than one p.p.m. it may be beneficial in the normal development of enamel of the teeth in both animals and man, according to Dean, Jay, Arnold, McClure, and Elvove (1939); Dean, Jay, Arnold, and Elvove (1941); Cox (1940); and Cox, Matuschak, Dixon, Dodds, and Walker (1939).

Although the toxicity of fluorine has only been recognized in recent years, its toxicology has been rather thoroughly investigated, and it is almost universally recognized as a general protoplasmic poison. The toxicity of fluorine for human beings was first recognized in a form of mottling of the enamel of teeth. When one mg. of fluorine is present per liter (one p.p.m.) of drinking water, there occurs a mild mottling of the enamel of the teeth of about 10 per cent of the children who have consumed it, up to the age of 10 years. While such mottling is a toxic manifestation resulting from fluorine, very little if any damage is done to the teeth. When one and one-half to two mg. per liter (one and one-half to two p.p.m.) of fluorine is found in drinking water, the percentage of children whose teeth become mottled is increased to about 50 per cent and the mottling so produced ranges from very mild through mild to moderate and, in a very small percentage of the cases, there may be a moderately severe effect. In the more severely affected cases the enamel of the teeth evidences poor structure and the mottling is very undesirable owing to disfiguration of the teeth. When concentrations of fluorine greater than two mg. per liter (two p.p.m.) occur in the drinking water of children, very severe damage is caused to the teeth of most of them. Extensive

experimental investigations have been carried out on animals in order to evaluate the toxic effects of many of the salts of fluorine. These investigations have been stimulated primarily by the extensive use of cryolite in spray materials and of rock phosphate as a source of mineral supplements to the diets of both animals and man. It has been conclusively proved that even when the so-called insoluble salts of fluorine are administered in the diet of animals at very low levels on the basis of their fluorine content there is no difference between the toxicity of them and the more soluble compounds.

The bleaching of the teeth of rats when fluoride is ingested in the drinking water or the diet corresponds to mottling of the enamel of the teeth of children who have ingested drinking water or food containing fluorides. When bleaching is used as a criterion for the toxicity of ingested fluorine, the rat is not less than from seven to 14 times more resistant than children between the ages of two to 10 years. The teeth of adults do not become mottled from exposure to fluorine in drinking water or in food. Water containing fluorine is widely distributed throughout the United States, and the water supply of a large number of communities contains one mg. or more of fluorine per liter. The intake of fluorine by cattle, rats, and dogs interferes with the normal metabolism of the tissues of the body which manifests itself in disturbances of growth, health, and bone and tooth structure and in the case of the teeth it causes histological changes in the cells whose function is the formation of enamel. In order that the reader may obtain more detailed discussions of the points mentioned above concerning the toxicology of fluorine, a few references to the literature are presented, from the bibliographies of which other references may be obtained for detailed discussion: Dean, Jay, Arnold, McClure, and Elvove (1939); Dean, Jay, Arnold, and Elvove (1941); Cox (1940); Cox, Matuschak, Dixon, Dodds, and Walker (1939); Roholm (1937); Greenwood (1940); DeEds (1933); Smith, Lantz, and Smith (1931); and Smith and Smith (1940).

MERCURY

The indications are that mercury is quite widely distributed in plant and animal tissues but the quantities which naturally occur are extremely small. Analyses of normal fresh tissues of man, for example, show .1 to 1.0 micrograms per 100 grams (.001 to .01 p.p.m.) of those tissues in which storage does not seem to occur. According to Stock (1940) storage occurs in kidney (4 to 10.2 micrograms per 100 grams), in liver (1.2 to 11.8 micrograms per 100 grams), and in pituitary (4 to 13.3 micrograms per 100 grams). Nelson and Laug (1941) have made analyses of normal tissues of small animals (rats, rabbits, etc.) by a very specific and sensitive method and have shown that mercury is present only in traces. There is no evidence from the literature that mercury is required in nutrition. Because of its very marked deleterious effect on tissues, its toxicology, however, has been extensively investigated. When mercury is employed in industrial processes, it is well known that extreme care must be used to prevent its inhalation or ingestion. Because of its appreciable vapor tension and the ease with which it can enter into chemical combination, the metal itself is poisonous as well as its soluble salts. Even in the form

of its most insoluble compounds it has caused poisoning. As an example of one of many cases which may be found in the literature, when workmen were exposed to 12 to 85 mg. per 10 cubic meters of air, numerous symptoms of intoxication occurred, and poisoning has occurred when the air contains as little as .6 mg. per 10 cubic meters, according to the Ohio Department of Health (1940). The symptoms of chronic mercury poisoning begin with gingivitis, stomatitis, and renal irritation and are followed by severe kidney injury and other miscellaneous symptoms.

LEAD

Lead is ubiquitous in its distribution in plant and animal tissues, and the concentration is such, according to numerous investigators, that the amount ingested daily by man on an average diet is approximately .3 mg. According to Kehoe, Cholak, and Story (1940a,b), who have made extensive analyses of foods which comprise the human dietary, the freely chosen human diet varies considerably in its lead content from day to day, and the increment of lead content above that which is present naturally is due to contamination from a variety of sources. Average blood lead values obtained by different investigators for normal individuals is about 30 micrograms per 100 c.c. with by far the largest percentage falling within the range of 20 to 50 micrograms per cubic centimeter. A similar figure is obtained per liter of normal urine. Even though lead is so widely distributed in plant and animal tissues, there has been no indication of any possible value in nutrition. Because of the fact that the toxicity of lead has long been recognized, there are more than 10,000 references to it in the literature. Only in recent years, however, have carefully controlled experimental investigations been made concerning its toxicity when it is included in the diet in very small quantities. Like mercury, fluorine, and some of the other elements, its chief hazard is due to its cumulative properties. Because of the fact that it is not an acute irritant to the gastrointestinal tract and no deleterious effects are noticed during the early course of its ingestion, exposure may occur for a considerable period of time before the severity of its effects are manifested. From 90 to 95 per cent of the lead ingested by human beings is excreted in the feces. Of the remainder about half is excreted in the urine, while the other half is retained by the body, and the same holds true for animals.

From experiments on animals the following facts, among others, have been ascertained:

1. The assimilability of water-soluble lead compounds is not greatly different from that of the so-called insoluble lead compounds when these are administered at low levels.

2. When relatively large amounts of dried apple are added to the diet there is somewhat less storage of lead in the animal tissues than on a similar diet that does not contain dried apple.

3. There is a small decrease in the amount of lead stored when the same salt of lead is ingested with the food as when ingested with the drinking water.

4. Lead can be transmitted to the young in the milk of the mother.

5. Young animals store much more lead and are much more susceptible to lead poisoning than are adult animals, and there is wide variation in the susceptibility of individual animals of the same species as well as a wide variation in the response of different species.

6. Animals on a low-calcium diet absorb and store more lead and are more susceptible to lead poisoning than are animals on a high-calcium diet.

Clinical and experimental data obtained on man show a close correlation with the data obtained on animals, for example:

1. When lead is added experimentally to the diet of man, there is storage in the tissues of about five per cent of the amount ingested.

2. Children are more susceptible to lead poisoning than adults.

3. There is wide variation in susceptibility to lead poisoning.

4. Lead poisoning has occurred in human beings from the so-called insoluble lead salts as well as lead in drinking water and other beverages.

5. Lead poisoning is particularly difficult to diagnose because of the variability in the symptoms and because of their similarity to symptoms of other forms of disease or intoxication.

6. Under certain abnormal physiological conditions, such as acidosis or alkalosis brought on by disease or clinical treatment, as well as through the use of certain types of drugs, lead is released from the places where it is stored in the body and causes a rise in the blood and urinary lead, and lead intoxication may occur. A few of the most recent references to the literature are given and from these, earlier references may be obtained —Calvery (1938); Neal, Dreessen, Edwards, Reinhart, Webster, Castberg, and Fairhall (1941); Shields, Mitchell, and Ruth (1940); McCann and Vogt (1933); Aub, Fairhall, Minot, and Reznikoff (1926); Lederer and Bing (1940); and Minot (1938).

ARSENIC

Analyses of a large number of foods have shown that the average content is less than one p.p.m., with a few natural foods showing nondetectable amounts and even a much smaller number exhibiting quantities exceeding 10 p.p.m. As an example, the analyses, summarized by Beeson (1941), of 56 items showed an average arsenic content of .623 p.p.m. on a dry-weight basis. According to unpublished data from the U. S. Food and Drug Administration (1930-41) analyses of hundreds of other samples on both fresh and dry-weight basis have shown that by far the majority of them contain less than this amount. Although the suggestion has been made that arsenic may play a role in normal biological processes, there is no specific and conclusive proof that this is the case.

The toxicology of arsenic has been almost as completely investigated and there are almost as many references to it in the literature as for lead. This is principally because of the fact that it has had much wider therapeutic use than have the salts of lead. Arsenic compounds often cause acute toxicity but, like lead and mercury and some of the other elements, its chronic toxicity for man is of far greater significance. When administered to both animals and men, even in its most insoluble forms, it is soluble in the secretions of the gastrointestinal tract and is absorbed, some

of it being stored in the tissues but the greater portion being excreted in the urine. The storage is principally in liver, spleen, muscle, skin, hair, and brain and that stored in the brain tissue remains more constant over a period of time than that stored in the tissues of other vital organs. As a result of the storage of arsenic in the tissues of the body, conditions directly attributable to it, namely, pigmentation, dermatitis, exfoliation, neuritis, hyperkeratosis, and various forms of cancerous conditions of the skin, have been observed years later. There is wide individual variation in the physiological response to arsenic in both man and animals. Some persons become sensitized after exposure to it so that subsequent exposures produce more marked toxic manifestations than the first exposure. A short bibliography is presented from which the reader may obtain further references to the literature: Underwood (1940); Calvery (1938); Neal, Dreessen, Edwards, Reinhart, Webster, Castberg, and Fairhall (1941); Ecker and Kernohan (1941); and Franseen and Taylor (1934).

ANTIMONY

Analytical data concerning the occurrence of antimony in plant and animal tissues is so meager that specific data concerning the amounts are not available, and as a result there are no data concerning its nutritive significance. Its physiological actions and its toxicology resemble those of arsenic. The chief difference is in its greater local irritation and less ease of absorption. When administered in small doses (four mg.) it acts as a depressant expectorant. In larger doses (30 mg. of tartar emetic) it acts as an emetic and causes nausea and vomiting. It has fallen out of favor for this use, however, because its action is too slow and it is too depressing. Fatal results have occurred after 150 mg. of tartar emetic. When administered in small quantities over a period of time, sufficient quantities are absorbed to cause chronic toxicity and the results are very similar to those in chronic arsenic poisoning. Its presence in food containers is sometimes encountered in the glazes for enamelware and occasionally in pottery glazes, but according to Sollmann (1936) and Wieland and Behrens-Heidelberg (1927), it should never be used for these purposes because its solubility is sufficient to present a health hazard.

BARIUM

Barium has been found to occur only occasionally in foods and tissues, and sufficient quantitative analyses have not been made to enable one to draw any conclusions as to the average amount present. Analyses of nine items of food presented by Beeson (1941) showed an average of 11 p.p.m. on a dry-weight basis. It is interesting that, according to Godden (1939) and Sheldon (1934), it has invariably been found in the choroid of the eye of various species and that the amount increases with age. There is no evidence of its necessity in life processes. Its chronic toxicity has not been very extensively studied but some investigations are under way in our own laboratory at the present time. Complete details of this investigation will be presented when the work is finished. The LD 50 doses of barium chloride for rats, mice, and guinea pigs have been found by Woodard and Calvery (1941) to be 118, 430, and 76 mg. per kg. respectively.

BROMINE

Bromine is invariably found in small quantities in practically all foods, and analyses, summarized by Beeson (1941), of 40 different foodstuffs, for example, have shown that the average content on a dry-weight basis is 4.5 p.p.m. The average content of fresh foods will, of course, be less. In some of the crustacea it forms an integral part of one of the amino acids present in the protein of these animals. It is likewise invariably found in the blood of various species and in human tissues, but there is no certainty yet that it has a specific function in the tissues of the higher forms of life, although suggestions have been made by Godden (1939) and Sheldon (1934) to that effect. The salts of bromine are widely used in therapeutics, and intoxication has been common, according to Hanes and Yates (1938), Gundry (1939), and Curran (1938), primarily because of its wide use in certain proprietary preparations. The syndrome of bromism in man is difficult to reproduce in animals, and although the chronic toxicity of bromides has been rather extensively investigated in experimental animals by Smith and Hambourger (1936), the results are difficult to interpret in terms of their effects on man.

SELENIUM

Selenium is one of the elements which does not occur widely distributed in plant and animal tissues but only in those foodstuffs which are grown in certain areas, particularly in the United States, and in the tissues of man and animals consuming foodstuffs coming from those areas. When foods containing selenium are consumed by man or animals, the selenium is distributed throughout all the tissues of the body and is present in eggs and milk from these animals in relatively high concentrations. The average content of selenium in foods is of little significance since it is entirely dependent upon the selenium content of the soil in which the food is grown and on the type of plant as well as the animal which consumes these. Certain grains, such as corn or wheat, grown on soils containing two p.p.m. may contain as high as 25 or 30 p.p.m. of selenium in the edible portion. Eggs from hens which have consumed such grains may contain on a fresh-weight basis as high as eight p.p.m., and the analysis of a batch of over 100 pounds of dried eggs from one of these areas showed a content of 22 p.p.m. on a dry-weight basis. There is no evidence that selenium is required in animal nutrition. On the contrary, there is a large amount of data to show that it is extremely toxic to the animal organism. When the tissues of animals which have been given selenium in their diets are examined, the first noticeable effect is on the liver; and on a low-protein diet containing as little as five p.p.m. of selenium, deleterious effects to the liver have been demonstrated by Morris, Fitzhugh, and Calvery (1941). The most extensive investigations of the toxicity of selenium have been made by Franke, by Moxon, and by M. I. Smith and their associates. A bibliography of the early work may be found in a publication by Moxon (1937). More recent publications of Moxon have appeared in *Poultry Science* and in the *Journal of Nutrition*. Smith's publications appear principally in the *Journal of Pharmacology* and *Experimental Thera-*

peutics. The toxicity of selenium for human beings has also been investigated by Smith, Franke, and Westfall (1936); and Lemley (1940).

TELLURIUM

The presence of tellurium has rarely been detected in foods and there is no evidence for its normal biological requirement. The investigations of its toxicology have shown that even at levels as low as 25 p.p.m. it was definitely toxic, as shown by its effect on growth, food consumption, and final weights of the animals. It was also found that the tellurites are more toxic than the tellurates. In addition to the above effects, it was noted by Franke and Moxon (1937) that there was considerable loss of hair from the animals.

MOLYBDENUM

The occurrence in foods and tissues and the biological significance of molybdenum has been so little investigated and there is so little agreement among investigators that it seems inadvisable to accord it any discussion. Its toxicity has been investigated by Franke and Moxon (1937) in comparison with several other elements and it was found that when it was included in the diet at a level of 50 p.p.m., it possessed about the same degree of toxicity as arsenic fed at the same level when the effect on growth and food consumption were used as criteria.

VANADIUM

When spectrographic analyses of food are made, the presence of vanadium is not always detected but, according to Underwood (1940), has been reported by some authors as appearing in minute traces. Although it has been shown to be required in some of the lower forms of animal life, there is no evidence of its requirement by the higher forms. Investigations, by Franke and Moxon (1937), of its toxicity when included in the diet, have shown that when it was included at a level of 20 p.p.m., it was about equal to the toxicity of tellurium, but at the level of 50 p.p.m., it was more toxic than tellurium at the same level.

THALLIUM

Like vanadium and some of the other elements, when foods are examined by spectrographic analysis, thallium is not always detected and when it does appear, it is not in amounts sufficient for quantitative determination. It has no known nutritive significance but is known to be highly toxic. For rabbits, for example, its acute toxicity has been shown, by Hofman (1937), to be 15 to 18 mg. per kg. One of its specific effects is its depilatory action, and it has been used therapeutically in children as a temporary depilatory in ringworm of the scalp, but because of the numerous cases of severe poisoning resulting, such use is not justified. In its toxicity it is more treacherous than lead, being an accumulative poison whose effects develop rather slowly with the development of severe nervous symptoms and death. The phenomena of chronic poisoning, according to Sollmann (1936), include stomatitis, gastroenteritis, diarrhea, loss of hair, peripheral neuritis, pain in the extremities, tremors of the hands, weakness of the muscles, general degenerative changes of the organs and tissues, and depression, dementia, and other neuroses.

SUMMARY

A review of the literature has furnished us with information concerning the occurrence, nutritional value, and toxicity of a number of elements which occur in small quantities in foods. With this information available we have been able to place these trace elements into three classes: nutritive trace elements—cobalt, copper, iron, iodine, manganese, and zinc; nonnutritive, nontoxic trace elements—aluminum, beryllium, boron, chromium, silicon, strontium, tin, titanium, and nickel; nonnutritive, toxic trace elements—silver, bismuth, cadmium, fluorine, mercury, lead, arsenic, antimony, barium, bromine, selenium, tellurium, molybdenum, vanadium, and thallium. In addition to the elements mentioned above, there are a few—caesium, cerium, germanium, gold, lanthanum, lithium, rubidium, thorium, and zirconium—the presence of which has been reported but for which sufficient quantitative data are not available. Their significance in nutrition is not established and their toxicology has not been thoroughly investigated. For these reasons they are being omitted from this discussion and, in addition, no claim is being made that this presentation is entirely complete. Besides the six nutritive elements mentioned above, it should be mentioned in conclusion that although physiological significance has been ascribed to arsenic, aluminum, barium, bromine, fluorine, nickel, rubidium, and silicon, entirely satisfactory demonstration of their essential nature for higher animals has not yet been accomplished except possibly in the case of fluorine.

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DETERMINATION OF VITAMIN C AND ITS SAMPLING VARIATION IN STRAWBERRIES[1]

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The importance of vitamin C content of fresh fruit in the human diet is well recognized. Strawberries have been found to be a rich source of vitamin C—Kirk and Tressler (1941), Kohman, Eddy, and Halliday (1928), and Satterfield and Yarbrough (1940). This is particularly significant since strawberries ripen in the spring when other fresh fruits are not plentiful. Large variations in vitamin C content within varieties of strawberries have been reported by Kirk and Tressler (1941) and Satterfield and Yarbrough (1940). No explanations for these variations have been given.

It seemed necessary, therefore, to study the conditions affecting the vitamin C content of strawberries. Herein is reported a satisfactory sampling procedure and an improved method of extraction which are to be employed in subsequent studies concerning varietal variations in vitamin C content of strawberries and effects of environmental factors.

METHODS OF EXTRACTION

Previous investigators have used a mortar and pestle with sand to grind the strawberries. This method proved to be inadequate because of the time involved in grinding the strawberries to a fine pulp and because of the decomposition of the ascorbic acid during the long period of grinding. Therefore, a motor-driven, emulsifying blender has been used in the present study. It consisted of a glass container in the bottom of which were fixed four clover-leaf pattern blades which rotated 1,750 times per minute. A 25-gram sample was placed in the container and 75 c.c. of a mixture of four per cent of metaphosphoric acid and eight per cent of acetic acid were added. One minute was required to beat the ripe fruit into a very fine pulp. This is a time-saving method and a uniform extraction is always obtained. The extract was centrifuged for 15 minutes and titrated immediately with an electrometric titrimeter, according to Kirk and Tressler (1939), using sodium 2, 6 dichlorobenzenindophenol of known ascorbic acid values as an indicator. The dye was standardized each day and the contact points of the electrodes were kept free from the interference of dirt and grease.

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CONDITIONS AFFECTING METHODS OF SAMPLING

The ascorbic acid values of five samples drawn from a commercially packed quart of strawberries are given (Table 1). Determinations were made in duplicate on each sample. These values range from 35.9 to 44.8 mg. per 100 grams of fruit, a variation within the quart of 22 per cent in ascorbic acid content. If this represents the random variation one must expect in a quart of berries from one variety and one location, a very large number of samples will be required to detect reasonable differences between different varieties or different environments.

This variation from sample to sample within a given quart of berries seemed extremely large to the investigators; therefore, they began to check possible sources of variation. It seemed advisable to make several determinations to see if there were indications that similar differences existed in ascorbic acid content between strawberries ripened in the sun and those

TABLE 1

Variation in Ascorbic Acid Content Within a Quart of Commercially Packed Klondike Strawberries[1] *(Chadbourn, N. C.)*

Sample	Ascorbic acid at extraction
	mg./100 gm.
A	44.8
B	35.9
C	40.6
D	39.0
E	41.8
Mean	40.4

[1] Picked May 1; analyzed May 2, 1941.

ripened in the shade. It has been found by Harding, Winston, and Fisher (1941), for example, that oranges ripened in sunshine are significantly higher in ascorbic acid content than fruit ripened in shade. The ascorbic acid content of the portion of apple fruit exposed to sunshine has been found, by Murphy (1928), to be greater than that of the shaded portion.

Therefore, samples of uniform-size strawberries (about 16 berries per 100 gm.) with the same degree of ripeness were selected from shaded and exposed locations in the same field. The results (Table 2) show a slightly higher ascorbic acid value for the samples of strawberries grown in the sunshine than for those ripened in the shade.

Possible effects of soil variation on ascorbic acid variation are shown (Table 3). Fields A and B were experimental fields in which all the plots sampled received the same fertilizer treatments. The differences in ascorbic acid content of fruit from the various plots within Fields A and B represent possible effects of soil variation. Fields C, D, E, and F were growers' fields and received fertilizer treatments similar to those in A and B. As the data show, the variation was from 36.3 mg. per 100 grams of fruit in Field E to 52.2 mg. per 100 grams of fruit in Field C.

The data show a close agreement between duplicate quarts sampled under the same conditions as to location, time, degree of maturity, expo-

TABLE 2

Ascorbic Acid Content of Sun-Ripened and Shade-Ripened Klondike Strawberries [1] (Chadbourn, N. C.)

Sample	Ascorbic acid content	
	Quart 1	Quart 2
Sun-ripened [2]	*mg./100 gm.*	*mg./100 gm.*
A	45.6	46.7
B	45.9	45.9
C	45.0	46.4
Shade-ripened [3]		
A	42.5	42.5
B	43.4	41.7
C	42.0	41.4
Densely shaded [4]	35.6	35.6

[1] Picked May 7; analyzed May 8, 1941. [2] Sun-ripened fruit were exposed to direct sunshine during ripening. [3] Shade-ripened fruit were those which ripened under the foliage of the plants. [4] Densely shaded fruit were those which ripened under a strawberry cup.

TABLE 3

Ascorbic Acid Content of Klondike Strawberries From Different Fields [1]

Field	Ascorbic acid content	
	Quart 1	Quart 2
Field A [2]	*mg./100 gm.*	*mg./100 gm.*
Plot 1	47.8	47.1
Plot 5	48.0	47.1
Plot 6	48.6	47.8
Plot 7	50.0	49.3
Field B [2]		
Plot 1	46.4	47.1
Plot 2	43.5	44.2
Plot 3	40.6	41.3
Plot 4	46.4	45.6
Plot 5	50.1	48.3
Plot 6	45.0	46.5
Plot 7	47.1	47.8
Plot 8	48.5	50.1
Field C	51.5	52.2
Field D	47.1	47.8
Field E	37.7	36.3
Field P	40.2	42.0

[1] Picked May 17; analyzed May 18, 1941. [2] Fields A and B were experimental fields in which the plots received the same fertilizer treatments. Fields C, D, E, and F were growers' fields and received similar fertilizer treatments.

Note: Credit is due Prof. Gertrude Cox and Prof. J. A. Rigney of the Department of Experimental Statistics, North Carolina Agricultural Experiment Station, for suggestions concerning the selection of samples.

sure to sunshine, and size of fruit (Tables 2 and 3). It would appear, therefore, that reliable samples have been secured and that by adopting uniform standards for these factors rather small differences between varieties or fertilizer treatments may be detected. This method of careful selection of samples was used in all subsequent determinations.

The range from 36.3 to 52.2 mg. of ascorbic acid per 100 grams of strawberries grown in different fields (Table 3) shows that different environments markedly affect the vitamin C content of strawberries. Therefore, in comparing the ascorbic acid content of different varieties of strawberries, or in studying the effect of different treatments on the ascorbic acid content of a single variety, considerable precaution should be exercised to eliminate these other sources of variation. In order that samples may be comparable, they should be taken from the same environ-

TABLE 4

Effect of Variety on Ascorbic Acid Content and pH of Ripe Strawberries[1] *(Willard, N. C.)*

Variety	pH of fruit	Ascorbic acid content of fruit		
		Quart 1	Quart 2	Quart 3
		mg./100 gm.	*mg./100 gm.*	*mg./100 gm.*
Fairmore	3.90	66.4	65.8	65.7
Missionary	3.39	46.5	45.8	45.8
Massey	3.58	41.8	41.4	41.8
Blakemore	3.26	32.2	33.0	32.6

[1] Picked May 14; analyzed May 15, 1941. Samples for the varieties determinations were collected by E. B. Morrow, Associate Horticulturist, North Carolina Experiment Station.

ment, represent similar exposure to the sun, and be of approximately the same size and degree of ripeness.

VARIETAL DIFFERENCES IN ASCORBIC ACID CONTENT

The data show that the Fairmore variety has the highest ascorbic acid value of the varieties analyzed in this study (Table 4). This is in agreement with the findings of Satterfield and Yarbrough (1940). There is close agreement among the three quarts of the respective varieties in ascorbic acid content; however, there was a marked difference in the varieties ranging from 32 to 66 mg. per 100 grams of fruit. Satterfield and Yarbrough (1940) reported a range in average values for their respective varieties of 36 to 65 mg. per 100 grams of fruit. Kirk and Tressler (1941) have shown a range in average values for 11 varieties of 40 to 104 mg. per 100 grams of strawberries. They reported high vitamin content for the Beacon variety (104 mg.), Southland (93 mg.), Redheart (84 mg.), Fairfax (68 mg.), and Dorsett (59 mg. per 100 grams of fruit). They noted the high day-to-day variations in vitamin C content— 32 mg. on June 18 to 83 mg. on June 26 for Dorsett, and 102 mg. on June 28 to 40 mg. on July 5 for Fairfax.

The portion of the fruit sampled is a factor in the variation of ascorbic acid in strawberries (Table 5). The outer portion of the Blakemore contained 20 per cent more ascorbic acid per 100 grams of fruit than did the center portion of the same berries. The outer portion of the Fairmore

strawberry contained nine per cent more ascorbic acid per 100 grams of fruit than did the center portion.

The results show that the ascorbic acid content is only approximately half as high in very green fruit as in ripe fruit on a weight basis (Table 6). On a fruit basis, the green berries had only 20 per cent of the vitamin C found in the ripe fruit.

TABLE 5

Ascorbic Acid Content of Inner and Outer Portions of Ripe Strawberries[1] (Willard, N. C.)

Variety	Ascorbic acid content	
	Quart 1	Quart 2
Fairmore	*mg./100 gm.*	*mg./100 gm.*
Center portion [2]	78.3	77.5
Outer portion [3]	86.7	86.0
Blakemore		
Center portion [2]	40.0	40.7
Outer portion [3]	49.2	50.0

[1] Picked May 8; analyzed May 10, 1941. [2] Center portion is equivalent to one-third of the fruit and is anatomically the medulla or fleshy pith of the receptacle. [3] Outer portion is equivalent to two-thirds of the fruit and is anatomically the cortex of the receptacle.

TABLE 6

Effect of Degree of Ripeness on Ascorbic Acid Content of Klondike Strawberries[1] (Chadbourn, N. C.)

Degree of ripeness	pH	Ascorbic acid content		
		Quart 1	Quart 2	Per 100 berries
		mg./100 gm.	*mg./100 gm.*	*mg./100 gm.*
Ripe [2]	3.50	46.7	45.9	280
Pink green [3]	3.05	32.0	31.3	177
Very green [4]	2.99	23.3	24.0	59

[1] Picked May 8; analyzed May 9, 1941. [2] One hundred ripe berries averaged 605 grams fresh weight. [3] One hundred pink green berries averaged 559 grams fresh weight. [4] One hundred very green berries averaged 244 grams fresh weight.

SUMMARY

Ascorbic acid (vitamin C) in strawberries was efficiently extracted in one minute with a metaphosphoric acid mixture employing an emulsifying blender.

Considerable variation was found in ascorbic acid content of commercially packed strawberries.

The ascorbic acid content of sun-ripened strawberries was greater than that of berries ripened in the shade.

In Klondike strawberries grown in different fields the ascorbic acid content varied from 36 to 52 mg. per 100 grams of fruit.

Careful selection of samples was found to be necessary.

Varieties of strawberries grown in the same field at the same fertility level ranged from 66 to 32 mg. of ascorbic acid per 100 grams of fruit and ranked in the following descending order as to ascorbic acid content: Fairmore, Missionary, Massey, Blakemore.

The outer portion of the Blakemore strawberry contained 20 per cent more ascorbic acid per 100 grams of fruit than did the center portion, whereas the outer portion of the Fairmore variety contained nine per cent more ascorbic acid per 100 grams of fruit than did the center portion.

On the basis of a hundred Klondike strawberries, the ascorbic acid content increased from 59 mg. in green berries to 280 mg. in ripe berries.

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AN ANALYTICAL STUDY OF CUCUMBERS AND CUCUMBER PICKLES[1]

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There is relatively little information on the nutritive value of cucumbers and still less on pickles made from them. In some of the earlier textbooks and papers on nutrition, pickles are listed as indigestible and harmful. This view is still widely held so that it was considered feasible to extend our knowledge of the composition of cucumbers and cucumber pickles in order that a correct dietary value might be assigned to these foods.

Pickles may be classified under three general types, according to Fabian and Switzer (1941). Type 1 is the fermented fresh cucumber pickle, such as genuine dill pickles. Type 2 is the unfermented fresh cucumber pickle—fresh cucumbers packed in glass containers to which is added a flavoring liquor. The jars are then sealed and pasteurized immediately so that no fermentation takes place. "Bread and butter" and fresh pasteurized dill pickles are examples of this type. Type 3 pickles are made by first salting the fresh cucumbers in a salt brine which is gradually increased in strength until it reaches approximately 15 to 16 per cent salt. During the salting period the pickles undergo a lactic acid fermentation and a curing process. After they have been completely cured, they are known as "salt-stock." When they are ready to use, they are processed by soaking them in cold or hot water until most of the salt has been removed. At this stage they are called "freshened or processed stock." The freshened pickle is then "finished" by the addition of a suitable liquor. Examples of this type of pickle are sour, sweet, processed dill, and relish. There are many other kinds.

This study was made over a three-year period. At least two of each different type of pickles briefly outlined above were studied. Included in this work was the influence of the type of soil, such as clay, sandy loam, and muck, on the chemical composition of the cucumbers and pickles. In order to make the work as comprehensive as possible, samples of different types of pickles were obtained for analysis from manufacturers located in widely separated geographical parts of the country, such as California, Canada, Connecticut, and Georgia. All the pickles were made under commercial conditions in a large pickle factory so as to be representative of the pickles purchased on the market.

Chemical and biochemical analyses were carried out with samples of the fresh cucumbers which were to be made into pickles. During the processing samples were taken and the same analyses repeated. In this way

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it was possible to follow the changes that were taking place and study the influence of the different operations on the composition of the pickle.

EXPERIMENTAL PROCEDURE

Moisture was determined by weighing a 10-gram sample of material into a previously weighed crucible and drying for three to four hours at 80°C.(176°F.) in a vacuum oven until the weight was constant.

Ash was determined by the official method of A. O. A. C. (1940) XXVIL, 8.

Total nitrogen was determined by a combination of the Kjeldahl and Gunning methods (1940) II, 21 and 22. A slight modification of the Kjeldahl method was used by distilling the ammonia into a four-per cent boric acid solution instead of the standard acid solution, and using a factor of 6.25 to convert the nitrogen into per cent of protein.

Crude fat or ether extract was determined by the official A.O.A.C. (1940) direct method XXVII, 21 and 22.

Simple carbohydrates. The reducing sugars were determined by the official A.O.A.C. method (1940) as given in XII, 2b and 42; XXXIV, 37, 38, and 40.

Carotene. Two different methods were employed for the determination of carotene. In 1939 the Peterson, Hughes, and Freeman (1937) method was used for determining the carotene content of the genuine dill pickles packed that year. The photelometer was used and was equipped with a blue filter supplied by the Central Scientific Company. In 1940, the method developed by Petering, Wolman, and Hibbard (1940) was used to determine the carotene content of all the cucumbers and pickles analyzed. The conversion factor used to change carotene into the vitamin A equivalent was .0006 milligram, the amount of pure beta carotene which possessed activity equal to one International or one United States pharmacopoeial unit of vitamin A.

Vitamin B_1, the antineuritic vitamin, was determined biologically with rats. The standard used for the controls in these experiments was the product made by Jansen of Amsterdam, Holland, which was identical in character and potency with the International Standard.

Vitamin B_2 or G. The growth method with rats was also employed in determining the amount of vitamin B_2 in cucumbers and pickles. The standard used for the control feeding experiment was autoclaved brewer's yeast made by Harris.

Vitamin C or the antiscorbutic vitamin. A chemical method was used for this determination. The material was extracted with a mixture of 16 per cent trichloroacetic and six per cent metaphosphoric acids and titrated with sodium 2.6 dichlorophenol indophenol.

Vitamin D. The vitamin D or antirachitic vitamin was determined according to U.S.P. line-test method.

Calcium was determined according to the official method (1940) XII, 10 of the A.O.A.C.

Copper. An unpublished modification, by L. I. Butler of the Michigan State Experiment Station Chemistry Section, of the Callan and Henderson method (1929) was employed. The colorimeter and also a photelom-

eter were utilized, and the copper concentration was obtained from a previously constructed transmission-concentration curve.

Iron. The titrimetric method (1940) XII, 8, of the A.O.A.C. was used for determining the amount of iron present in cucumbers and pickles.

Phosphorus. Phosphorus was determined by slightly modifying the Youngblood and Youngblood method (1930) used for blood.

INFLUENCE OF TYPE OF SOIL ON DILL PICKLES

A study was made over a two-year period, 1939 and 1940, of the influence of the type of soil on composition of the cucumbers and the pickles made from them.

Five bushels of cucumbers, varying in size from 1,000 to 1,200 per 45-gallon cask, that had been grown on three different types of soil—clay, muck, and sandy loam—were collected in the middle of the pickle season at a salting station. Chemical and biochemical analyses were made of representative samples of each lot of the fresh cucumbers. The rest of the cucumbers were made into dill pickles according to the following procedure: About three to four pounds of salt-cured dill was placed in the bottom of the barrel and 125 pounds of fresh cucumbers added. Another layer of about the same amount of dill weed was added and the barrel was filled nearly full with fresh cucumbers, after which a final layer of dill weed was added. One quart of mixed-pickle spices and one quart of 10-per cent vinegar were added during the packing of the cucumbers. The barrel was then completely filled with 17 to 18 gallons of eight-per cent salt brine, headed, and allowed to ferment. Analyses were made of pickles from each of the barrels at the end of three- and six-week intervals during the fermentation.

The results of the chemical and biochemical analyses at the different periods and for each type of soil are shown (Table 1) for the year 1939 and (Table 2) for the year 1940.

DISCUSSION OF RESULTS

The moisture content of the fresh cucumbers (Tables 1 and 2) grown on the three types of soils varied only slightly for each type of soil but there was approximately a one-per cent increase in 1940 over 1939. The moisture content of the dill pickles made from these fresh cucumbers varied but slightly, irrespective of the year in which the cucumbers were grown. The total solids of the fresh cucumbers varied inversely with their respective moisture content. There was an increase of the total solids in the dill pickles which was due to the absorption of materials from the pickling brine.

The ash varied directly with the amount of total solids and also with the amount of inorganic matter in the cucumber. There was a noticeable increase in the amount of ash in 1940 for the fresh cucumbers, while in the pickles the variations were not so great. Cucumbers grown on muck loam in 1939 and 1940 showed an increase in both total solids and ash as compared with those grown on the other types of soil.

The protein in the cucumber usually varied with the amount of total solids. In 1939, the total solids in the cucumbers was greater than in 1940.

TABLE 1

Analyses of Cucumbers Grown in 1939 on Sand, Muck, and Clay Soil and of Dill Pickles Made From Them

Analysis	Fresh cucumbers			Dill pickles						
	Sand	Muck	Clay	Date 1939	Sand	Change	Muck	Change	Clay	Change
	pct.	*pct.*	*pct.*		*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Moisture	93.990	93.590	94.340	10/6	92.593	—1.5	94.643	1.1	92.609	—01.8
Total solids	6.010	6.410	05.650	10/6	7.381	22.8	5.357	—16.4	7.342	030.0
Ash	0.659	0.726	00.490	10/6	4.732	616.1	3.213	342.6	4.347	787.1
Protein	1.001	1.010	1.162	9/15	0.660	—33.6	0.709	—29.8	0.707	—39.2
				10/6	0.615	—36.6	0.555	—45.0	0.664	—042.9
Simple carbohydrate	1.7720	2.2140	1.6120	8/29	0.0000	—100.0	0.000	—100.0	0.0000	—100.0
Fat	0.1370	0.1170	0.1280	10/6	0.2160	59.1	0.190	62.3	0.2250	75.8
Calcium	0.0226	0.0276	0.0168	10/6	0.0280	23.9	0.030	8.7	0.0290	—72.6
Phosphorus	0.0255	0.0268	0.0249	10/6	0.0175	—25.5	0.021	—22.4	0.0177	—26.9
Iron (mg./kg.)	13.9	8.4	11.10	10/6	9.50	—31.6	11.10	32.1	6.7	—39.6
Copper (mg./kg.)	5.0	3.0	3.75	10/6	6.71	34.2	8.84	188.0	5.0	33.3
Vitamin A[1]	260.0	270.0	290.00	8/29	490.00	88.4	360.00	33.3	610.0	75.8
				9/15	520.00	100.0	340.00	25.9	520.0	79.3
				10/6	490.00	88.4	400.00	48.1	460.0	58.6
Vitamin B_1	10.0	20.0	10.0	10/6	.....[2]	.....	.....[2]	.....	.....[2]	.....
Vitamin B_2	90.0	90.0	90.0	10/6	060.0	—33.3	60.0	—33.3	60.0	—33.3
Vitamin C	243.0	234.0	235.0	10/6	132.0	—45.5	135.0	—42.3	135.0	—42.5
Vitamin D	0.0	0.0	0.0	10/6	0.0	.....	0.0	.....	0.0	.....

[1] Vitamins are expressed in International units per 100 grams, except vitamin B_2 which is in Sherman-Bourquin units per 100 grams. [2] Rats would not eat pickles.

TABLE 2

Analyses of Cucumbers Grown in 1940 on Sand, Muck, and Clay Soil and of Dill Pickles Made From Them

Analysis	Fresh cucumbers 9/6/40			Dill pickles						
	Sand	Muck	Clay	Date 1940	Sand	Change	Muck	Change	Clay	Change
	pct.	*pct.*	*pct.*		*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Moisture	95.381	94.926	95.011	10/18	92.357	—31.7	93.194	—18.2	22.837	—22.9
Total solids	4.618	5.074	4.989	10/18	7.643	65.5	8.806	34.1	7.163	43.6
Ash	0.274	0.438	0.381	10/18	4.166	1416.8	3.861	781.5	3.742	862.1
Protein	0.848	1.060	0.731	9/27	0.805	—5.0	0.726	—31.5	0.727	—0.5
				10/18	0.794	6.4	0.689	—35.0	0.721	—0.5
Simple carbohydrate	1.6300	1.5900	1.580	9/10	0.0000	—100.0	0.0000	—100.0	0.0000	—100.0
Crude fat	0.1580	0.1560	0.137	10/18	0.2180	38.0	0.2150	37.8	0.2000	46.0
Calcium	0.0175	0.0275	0.030	10/18	0.0268	51.4	0.0259	5.8	0.0198	—34.0
Phosphorus	0.0262	0.0300	0.026	10/18	0.0237	9.5	0.0212	—29.3	0.0286	10.0
Iron (mg./kg.)	13.9	13.9	12.0	10/18	8.7	37.4	6.7	—51.8	5.5	54.2
Copper (mg./kg.)	1.8	2.8	2.7	10/18	6.2	24.4	8.5	203.5	6.4	137.0
Vitamin A[1]	216.0	266.0	250.0	9/27	350.0	62.0	350.0	31.5	358.0	43.2
				10/18	335.0	55.0	442.0	66.1	412.0	64.8
Vitamin B_1	8.0	8.0	8.0	10/18	1.0	—87.5	2.0	—75.0	1.0	—87.5
Vitamin B_2	25.0	30.0	35.0	10/18	12.0	—52.0	15.0	—50.0	15.0	—57.1
Vitamin C	250.0	287.0	266.0	9/27	155.3	—37.6	127.0	—55.7	132.0	—50.5
				10/18	155.0	—38.0	110.0	—61.6	105.0	—60.5
Vitamin D	0.0	0.0	0.0	10/18	0.0	.....	0.0	.....	0.0	.....

[1] Vitamins are expressed in International units per 100 grams, except vitamin B_2 which is in Sherman-Bourquin units per 100 grams.

The type of soil appeared to have but slight influence on the amount of protein in the fresh cucumbers (Table 1). There is a greater variation, however, in the protein content of the fresh cucumbers grown in 1940 than in 1939. There is a decrease in the protein content of the dill pickles as compared with the cucumbers from which they were made, owing to the action of the pickling brine and subsequent fermentation which took place. The brine and products of fermentation dissolved the soluble proteins which diffused into the brine and served as food for the microorganisms present. The greatest variation in the protein content of the cucumbers and the dill pickles occurred in the cucumbers grown on muck.

The simple carbohydrate in the fresh cucumbers was slightly higher in 1939 than in 1940. This is to be expected since the total solids and the other constituents making up the total solids were likewise higher for 1939 than for 1940. The sugar had completely diffused out of the dill pickles within four days. This confirms the work of Fabian and Wickerham (1935) who found in their work on dill pickles that all the sugar had disappeared from the dill-pickle brine within six to eight days.

The amount of crude fat or ether extract increased in 1940 over that of 1939 in both the cucumbers and dill pickles. Results show that cucumbers grown on sandy loam had slightly more crude fat than those grown on the other types of soil (Tables 1 and 2). The crude fat of the pickles made in 1939 and 1940 showed an increase over the cucumbers from which they were made. There is a slight increase in the crude fat of the cucumbers grown in 1940 as compared with 1939.

The amount of calcium found in the cucumbers and the dill pickles made from them was fairly constant. The type of soil, the year in which the cucumbers were grown, and fermentation had but slight influence upon the amount present. This would indicate that it is in a stable form presumably constituting a part of the cell-wall structure of cucumbers.

Phosphorus was likewise fairly constant in amount in the cucumbers grown in the two different years. In all cases except one, there was a slight drop in the phosphorus content of the dill pickles as compared with the amount found in the cucumbers from which they were made.

Iron is considered a trace element in plants. The content of iron in 1939 varied slightly. The cucumbers grown on sandy loam had a trace more of iron than those grown on muck or clay soils. The results secured in 1939 checked exceptionally well with those obtained in 1940. The amount of iron found in the dill pickles was slightly less in all cases except one than the amount in the cucumbers.

The copper content of cucumbers in the three types of soil in 1939 showed some variation. Cucumbers grown in sandy loam contained the most copper. Next in amount were those grown on clay and least in amount were those grown on muck. The copper content of the dill pickles made from the cucumbers grown on the different types of soil was greater than that of the cucumbers. This increase may have come from the quart of 100-grain vinegar added to each barrel of dills or it may have been due to the higher total-solids content of the pickles as compared with the fresh cucumbers. Vinegar frequently contains a trace of copper, although the copper content of the vinegar used was not determined.

The carotene content of fresh cucumbers grown in 1939 in the three types of soil varied little in amount. The cucumbers grown on clay contained the largest amount of provitamin A (Table 1). Cucumbers grown on muck showed an intermediate value, and sandy loam cucumbers the lowest. The dill pickles made in 1939 showed a large increase in carotene after four days of fermentation and continued to increase until the sixth week, after which there was a slight decrease. In 1940 (Table 2) the carotene content of fresh cucumbers which were grown on three different types of soil showed only a slight variation from cucumbers grown on muck and clay loam, while those grown on the sandy loam soil did not contain as much. The dill pickles (Table 2) likewise showed an increase in carotene content during fermentation. On the whole, the provitamin A content of both the cucumbers and dill pickles for the two-year series compared exceptionally well. Attention should also be called to the fact that a different analytical method was used the second year.

In 1939 there was no difference in the vitamin B_1 content of cucumbers grown on sand and clay loam, whereas the muck cucumbers contained twice as much. It was not possible to determine the amount of vitamin B_1 in dill pickles for 1939, owing to the fact that the rats would not eat sufficient pickles. There was no difference in the vitamin B_1 content of fresh cucumbers grown in 1940 on three different types of soil. During fermentation there was a decrease in the vitamin B_1 content. It must be taken into consideration that because of its relatively low vitamin B_1 content a large amount of cucumber or pickle material must be fed. This limits the amount of basal diet ingested and restricts, thereby, the amount of growth. Loss of vitamin B_1 by withdrawal from the pickles could also contribute to the low values obtained.

The vitamin B_2 content was the same for fresh cucumbers grown on the three different types of soil in 1939. The subsequent pickling and fermentation caused a decrease in the amount of vitamin B_2, but the decrease was the same in all cases for all types of soil. Since vitamin B_2 is water soluble, doubtless some of it was dissolved and removed during the manufacture of the dill pickles. In 1940, the vitamin B_2 content of fresh cucumbers grown on different types of soil showed only slight variations, cucumbers grown on clay being highest and on sandy loam the lowest. The amount of vitamin B_2 was less in 1940 than in 1939, in both the cucumbers and the dill pickles made from them. Likewise in 1940, the manufacturing process affected the vitamin B_2 content to a greater extent than in 1939. Vitamin B_2 is a more stable compound than vitamin B_1, so that the factors most likely responsible for the decrease are water solubility and the removal of the vitamin from the pickle.

The amount of vitamin C in fresh cucumbers grown on different types of soil in 1939 and 1940 checked very closely. The values obtained were higher than those reported by Heinz (1939). The manufacturing process caused a loss of approximately 40 to 60 per cent of the vitamin C content. There was a gradual loss as the fermentation continued, the loss being comparable to that found in the conversion of cabbage into sauerkraut. Pederson, Mack, and Athawes (1939) found a loss of one-third to one-half the vitamin C content of kraut during canning and also holding it in the

vat after the fermentation was completed. During prolonged storage this loss may be even greater. The loss of vitamin C is greater if fermented and stored in small containers. Heinz (1939) found cabbage to contain 1,000 International units per 100 grams, while sauerkraut contained from 0 to 190.

There was no vitamin D present in either the cucumbers or pickles as judged by the method used.

INFLUENCE OF MANUFACTURING PROCESS

Salt-stock pickles. The first step in the manufacture of certain types of pickles, such as sweet, sour, mustard, and processed dill pickles, is the salting of the fresh cucumbers. It is necessary to salt and hold them for

TABLE 3

Analyses of Fresh Cucumbers, Salt Stock, Processed and Finished Pickles, Showing Influence of Various Manufacturing Operations on Composition

Analysis	Fresh cucumbers	Salt-stock pickles	Change	Sweet pickles	Change	Processed dills	Change
	pct.	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Moisture	95.000	90.167	—5.1	63.810	—32.8	93.3570	—1.7
Total solids	5.005	9.839	96.5	36.190	623.0	6.6430	32.7
Ash	0.519	6.481	1148.7	2.038	292.7	3.8330	638.5
Protein	1.427	1.038	—27.3	0.701	50.9	0.5335	62.6
Simple carbohydrate	1.1600	0.0000	—100.0	29.3000	2425.8	0.0000	—100.0
Crude fat	0.0580	0.1520	162.1	0.0380	—34.4	0.1280	120.6
Calcium	0.0315	0.0336	6.7	0.0114	—63.8	0.0138	—53.0
Phosphorus	0.0379	0.0288	—24.0	0.0233	—38.5	0.0151	—60.2
Iron (mg./kg.)	12.0	11.6	—3.3	3.0	—75.0	3.6	—70.0
Copper (mg./kg.)	1.8	8.4	36.6	6.5	261.1	8.3	361.1
Vitamin A[1]	166.0	600.0	261.4	409.0	146.38	492.0	196.38
Vitamin B_1	7.0	1.0	—85.7	0.0	—100.0	0.0	—100.0
Vitamin B_2	40.0	10.0	—75.0	10.0	0.0	12.0	20.0
Vitamin C	293.0	44.8	—84.7	44.8	0.0	40.0	—10.7
Vitamin D	0.0	0.0	.....	0.0	.....	0.0	.....

[1] Vitamins are expressed in International units per 100 grams, except Vitamin B_2 which is in Sherman-Bourquin units per 100 grams.

varying lengths of time (three months to three years or more) since the cucumber harvest season is only of four to eight weeks' duration.

The salting procedure for cucumbers used in making the salt stock for the sweet and processed dill pickles for the experiments was as follows: The fresh cucumbers were placed in a 600-bushel tank and, while the tank was being filled, a 7.85-per cent (30 degree salometer) brine solution of sodium chloride was added in a sufficient amount to completely cover the cucumbers. When the vat was filled, they were submerged in the brine by a false cover and permitted to ferment and cure for a period of about three months. During the curing process, salt was added at weekly intervals so that at the end of this period the brine tested 11.2 per cent by weight. The finished product is known as "salt stock." The influence of the salting of fresh cucumbers on their composition is shown (Table 3).

Sweet pickles are made from salt stock. The method used for the experiments reported here briefly was as follows: Salt stock was soaked in

tap water for 12 hours. This water was discarded and fresh water added to the pickles. The temperature of the water and pickles was raised to 54.4°C.(130°F.) and the pickles were allowed to remain in this water for one hour. The water tested 3.4 per cent salt (13 degrees salometer) at this time. The withdrawal of salt from the pickles is called "freshening" and the freshened pickles after heating are called "processed stock." The processed stock was made into sweet pickles by the addition of a combination of spice oils, 13 per cent distilled vinegar, and sufficient sugar to make the final test of the pickles and liquor 34.5 Brix (19 degrees Baumé). These ingredients were placed over the processed pickles and allowed to stand for 10 days. At the end of this time the pickles were sweet and tested two per cent acetic acid (20 grains), 29.3 per cent sugar, and two per cent salt and contained one part of spice oils to 4,600 parts of material; the analysis of sweet pickles is given (Table 3).

Processed dill pickles are also made from salt stock. The salt stock was freshened and processed the same as for sweet pickles. In these experiments enough salt stock was freshened to make both the processed dill and the sweet pickles. To the processed stock was added a brine consisting of vinegar, salt, and dill spice oil. They were allowed to stand in this brine two days after which the pickles tested .67 per cent acetic acid and 3.9 per cent salt (17 degrees salometer).

INFLUENCE OF SALTING

Salting fresh cucumbers according to the procedure outlined reduced the moisture content and increased the amount of total solids and ash (Table 3). The salting process also reduced the protein content and caused a complete disappearance of the sugars. The crude fat in the salt-stock pickles was increased by one-tenth per cent. There was a slight increase in the calcium content which may be due to the greater amount of ash in the salt stock than in the fresh cucumber. The phosphorus decreased after salting, and iron varied only a small amount. The copper content of cucumbers after salting increased about 6.5 per cent.

With regard to the vitamin content of the salt stock it was perhaps not surprising to find appreciable decreases in the amount of thiamin, riboflavin, and ascorbic acid. Withdrawal from the stock and instability undoubtedly account for the losses that occurred. In contrast a marked increase was observed in the carotene content of the salt stock. Changes of this type are unusual although Smith and Otis (1941) reported similar increases in the carotene content of carrots during storage.

The moisture content of the sweet pickles showed a great decrease owing mostly to the addition of sugar during their manufacture, while the moisture content of processed dill pickles increased slightly. The total solids increased accordingly in the case of the sweet pickle, whereas the total solids in processed dills decreased approximately 3.3 per cent, owing to the increase in moisture content which in this case was 3.5 per cent. There was a 67-per cent decrease in the ash of the sweet pickle as compared with the salt stock but a 300-per cent increase over that of the fresh cucumber. The decrease was due to the withdrawal of salt from the salt stock and the increase was due, in part at least, to the addition of sugar

to the processed stock. The amount of ash in the processed dill pickles was less because most of the salt was removed from the salt stock during their manufacture.

As can be seen from the table the changes with regard to the protein, fat, and inorganic elements other than those contributed by salt were of a small order. The carotene content of both sweet and processed dill pickles was less than that of the salt stock. The vitamin B_1 was already very low in the salt stock and practically none was demonstrable in the sweet and processed dill pickles. However, there seemed to be no loss of riboflavin or ascorbic acid during the processing and finishing of both types of pickles.

ANALYTICAL STUDIES ON FRESH CUCUMBER PICKLES

Fresh cucumber pickles are made from fresh cucumbers by the addition of acid, with or without sugar, salt, and spices. They differ from dill pickles in that they are not fermented and from sweet, sour, mustard, and processed dill pickles in that they are neither fermented nor processed. Two different types of fresh cucumber pickles were made and studied in this work.

Pasteurized dill pickles were made by washing fresh cucumbers and placing them in quart glass jars containing two sprigs of dill weed and one tablespoonful of whole mixed spices and then filling the jar with a brine consisting of salt, vinegar, water, and spice oils. It was found that the pickles were of such a size as to occupy 60 per cent of the volume of the jar and the rest of the space was filled with brine. The filled jars were then pasteurized at a temperature of 71.1 to 73.9°C. (160 to 165°F.) for 30 minutes and cooled immediately. After equilibrium had been established, the acetic acid content was .4 per cent and the salt 2.25 per cent.

Bread and butter pickles were made by slicing washed fresh cucumbers to a thickness of from five to seven millimeters and adding one pound of sliced onions for each 200 pounds of sliced cucumbers. These were then placed in a 6.6-per cent (25 degrees salometer) salt brine. For each 16 gallons of brined, sliced cucumbers and onions, 5.5 ounces of turmeric were added. They were allowed to remain in this solution from four to 12 hours after which they were dipped out and placed in 10-ounce jars. The jars were then filled with 2.25 ounces of a syrup consisting of 4.3 per cent acetic acid, 50 per cent sugar, and one part turmeric to 350 parts of the syrup. The jars were then vacuum-sealed and pasteurized at 76.7°C. (170°F.) for 25 minutes; the analyses of the fresh cucumbers, the pasteurized dill pickles, and the bread and butter pickles are given (Table 4).

INFLUENCE OF PROCESSING ON FRESH CUCUMBER PICKLES

A comparison of pasteurized dill pickles with the fresh cucumbers from which they were made shows that during the manufacturing process the moisture content decreased and the total solids increased by two per cent. There was a slight drop in the protein and a considerable reduction in the sugar content of the pickles. However, the reduction is not as great as in the salt stock where it all disappeared. There was a slight increase in crude fat.

The mineral constituents, such as calcium and phosphorus, are affected but slightly by manufacture, whereas iron and copper show increases which may be due to traces of these minerals in the salt and vinegar.

There was not as much change in the vitamin content of the pasteurized dill pickles as in the salt stock. As usual there was an increase in the carotene content in the manufactured pickle over that found in the fresh cucumber. The vitamin B_1 decreased but slightly. There was about a 40-per cent decrease in vitamin B_2 and a 20-per cent decrease in vitamin C. In general the vitamin destruction was far less in this type of pickle than in the salt-stock pickles.

TABLE 4

Analyses of Fresh Cucumbers and of Pasteurized Dill Pickles and Bread and Butter Pickles Made From Them

Analysis	Fresh cucumbers	Pasteurized dills	Change	Bread and butter	Change
	pct.	*pct.*	*pct.*	*pct.*	*pct.*
Moisture	95.177	93.756	—1.5	79.531	16.4
Total solids	4.823	6.243	29.4	20.469	324.4
Ash	0.543	2.342	331.3	2.372	336.8
Protein	1.067	0.744	—30.3	1.029	—3.6
Simple carbohydrate	1.4600	0.8600	—41.1	15.3000	947.9
Crude fat	0.1020	0.1100	7.8	0.0990	—2.9
Calcium	0.0206	0.0225	8.2	0.0300	44.2
Phosphorus	0.0275	0.0223	—18.9	0.0301	9.4
Iron (mg./kg.)	11.3	18.0	59.3	20.6	82.3
Copper (mg./kg.)	2.2	2.5	13.6	3.6	72.7
Vitamin A[1]	200.0	292.0	48.0	458.0	129.0
Vitamin B_1	8.0	7.0	—12.5	8.0	0.0
Vitamin B_2	25.0	15.0	—40.0	17.0	—32.0
Vitamin C	255.0	180.0	—29.4	200.0	—21.5
Vitamin D	0.0	0.0	.....	0.0	.....

[1] Vitamins are expressed in International units per 100 grams, except Vitamin B_2 which is in Sherman-Bourquin units per 100 grams.

There was a considerable change in the moisture, total solids, ash, protein, and simple carbohydrate content of the bread and butter pickles as compared with the fresh cucumbers from which they were made. These changes were due to the materials added to the cucumbers during their manufacture as well as the several processes through which they passed during manufacture. The reduced moisture, the increased total solids, ash, and simple carbohydrates were due to the addition of sugar, salt, acid, and turmeric.

There was an increase in all the minerals of approximately 50 per cent in the bread and butter pickles as compared with the fresh cucumbers. The increase is due to the increase in the ash content of the pickles over that of the cucumbers because of the addition of certain substances, such as sugar, salt, and vinegar.

The vitamin content of the bread and butter pickles compared very favorably with the vitamin content of the fresh cucumbers. There was much less change owing to the manufacturing process than in the case of salt stock. This indicates that the procedure used to convert the fresh

cucumbers into bread and butter pickles was less destructive to the water-soluble vitamins than other types of treatment, such as salting. There was approximately a 125-per cent increase in vitamin A, no loss of vitamin B_1, a decrease of about 30 per cent in vitamin B_2, and about a 20-per cent decrease in vitamin C. These decreases are considerably less than was the case for salt stock; the results are shown (Table 4).

CAROTENE AND ASCORBIC ACID CONTENT OF COMMERCIAL PICKLES

During the summer of 1941, samples of commercial pickle products were secured from various parts of the United States and from Canada. Because of the small amounts of thiamin and riboflavin found previously

TABLE 5

Analyses of Pickles From Various Sections of the United States and Canada[1]

Section of continent	Type of pickle	Carotene per 100 gm. (micrograms)	Calculated U.S.P. units vitamin A per 100 gm.	Calculated Int'l units vitamin C per 100 gm.
California	Salt stock	160.2	213.6	132
	Genuine dills	94.8	126.4	108
	Processed dills	49.8	66.4	180
	Sweet pickles	63.6	84.8	164
	Spiced pickles	87.0	116.0	114
	Fresh cucumber chips	72.6	96.8	156
Georgia	Salt stock	72.0	96.0	202
	Processed dills	73.2	97.6	186
	Sweet pickles	30.6	40.8	146
	Fresh cucumber pickles	103.2	137.6	122
	Fresh cucumber pickles, kosher	72.6	96.8	184
Connecticut	Salt stock	112.8	150.4	114
	Genuine dills	103.2	137.6	126
	Genuine dills—original brine	83.4	111.2	154
	Processed dills	42.6	56.8	146
	Sweet gherkins	47.4	63.2	182
	Sweet mixed	30.6	40.8	198
	Sweet mustard pickles	52.2	69.6	62
	Sweet relish	59.4	79.2	146
	Sour gherkins	20.4	27.2	132
	Sour mixed pickles	27.0	36.0	132
Canada	Salt stock	128.4	171.2	54
	Homemade sweet water	64.8	86.4	192
	Sweet mixed	76.8	102.4	164
Michigan	Fresh cucumber	92.4	123.2	236

[1] Analytical work done by W. H. Lynch.

in processed pickles it was decided to limit this part of the study to determinations of carotene and ascorbic acid. For the analysis of ascorbic acid the method used previously was again employed. The carotene, however, was extracted and determined by the convenient method of Moore and Ely (1941), the color being measured with the Cenco spectrophotelometer.

The pickles used in this study were derived from salt stock from the 1940 harvest except for three fresh-cucumber products designated as

fresh cucumber chips, fresh cucumber pickles, kosher and fresh cucumber pickles. The latter were made from the 1941 crop.

Because of the uncertainty of the biological equivalent of carotene values determined by physical methods and because of the significant increases that had been observed in the carotene content of finished pickles as compared with the fresh cucumbers, it was considered desirable to make a biological assay of one of the carotene extracts. This was done in the case of a sample of spiced pickles which had a fairly high carotene value. The official method for vitamin A determinations was employed and the results of this test indicated that the pigments contained in the extract had a biological value equivalent to 80 per cent of beta carotene. Although it must of course be recognized that there would be some variations in the distribution of the pigments in the extracts from the different types of pickle products, these variations would probably not be significant. Accordingly, the biological values assigned are based on the above-determined 80-per cent factor; these results as well as those for ascorbic acid are given (Table 5).

The results of these studies indicate that regional variations in the carotene and ascorbic acid content of cucumbers are smaller than the variations observed between different types of pickles from the same region. The particular type of processing employed involving the establishing of conditions more or less unfavorable for the preservation of certain dietary constituents largely determines the amounts of these available to the consumer at the time of purchase. Although not conspicuous as a source of certain food essentials, pickle products do nevertheless have value besides stimulating appetites. Credit should therefore be accorded to them for the organic and inorganic nutrients which they contain.

SUMMARY

There was an increase in the total solids and a decrease in the moisture content in practically all varieties of pickles as compared with the fresh cucumbers from which they were made. In the case of sweet pickles and bread and butter pickles these changes were especially pronounced owing to the materials added during their manufacture.

There was a definite decrease in the protein content of the various types of pickles as compared with the fresh cucumbers from which they were made.

The simple carbohydrates were removed during the first four days of fermentation in the two kinds of fermented pickles studied—genuine dill and salt-stock pickles.

In most cases the quantity of crude fat varied directly with the amount of total solids.

There was a reduction in the calcium and phosphorus contents of pickles as compared with the fresh cucumbers from which they were made. The reduction was greatest for processed dill pickles.

In most instances there was a slight loss of iron in the pickles owing to the manufacturing processes. However, there was an increase in the iron content in the case of pasteurized dill and bread and butter pickles.

In general there was an increase in the copper content of the pickles over that found in the fresh cucumbers from which they were made.

The carotene content of different lots of fresh cucumbers increased during their manufacture into pickles. The increases ranged from 23 per cent for pasteurized dill pickles to 261 per cent for salt-stock pickles.

There was a decrease in the amount of the other vitamins studied when fresh cucumbers were made into dill pickles. At the end of a six-week period a loss of about 50 per cent was observed in the thiamin, riboflavin, and ascorbic acid content.

The salting process produced the greatest loss of the water-soluble vitamins. Subsequent processing and finishing of the salt stock into sweet pickles or processed dill pickles caused little loss except in the case of carotene.

Excluding carotene, vitamin losses ranged from 12.5 to 40 per cent when fresh cucumbers were made into pasteurized dill or bread and butter pickles. However, these losses were less than for salt stock which ranged from 75 to 86 per cent and for dill pickles which ranged from 33 to 87 per cent.

A determination of the biological value of the carotene fraction extracted from a sample of spiced pickles indicated a value of 80 per cent of the color value expressed as beta carotene.

A study of the carotene and ascorbic acid content of commercial pickles from various parts of the United States and from Canada indicated wide variations in the case of carotene and smaller variations in ascorbic acid. Doubtless the variety of cucumbers, the climatic conditions, and soil fertility contribute to these variations, although it is obvious that the method of processing may be one of the major factors involved.

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BACTERIAL SPOILAGE OF ICED FRESH CRABMEAT

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The spoilage of fresh crabmeat packed in cracked ice has for many years been a vexing problem to the sea-food industry. The rapid deterioration of crabmeat stored at temperatures just above freezing necessitates a rapid turnover of the product to avoid losses. The purpose of the study here presented was to determine the bacterial species involved in the decomposition of crabmeat at low temperatures, and their growth characteristics at the temperatures of commercial storage of crabmeat.

Harris (1932) reported a bacteriological investigation of the spoilage of Maryland and Virginia crabs and crabmeat held at various temperatures. He concluded that the *Proteus* group was the most important in spoilage, with the genera *Pseudomonas* and *Flavobacterium* possibly contributing to the deterioration. Other genera isolated were *Streptococcus*, *Sarcina*, and *Achromobacter*.

Harris (1932) and Tobin and McCleskey (1941) observed that the pH of fresh crabmeat was about 7.2 to 7.4, while in spoiled crabmeat the pH value was usually 8.0 to 8.5. Tobin and McCleskey (1941) noted that crabmeat artificially acidified to pH 5.1 to 5.9, by dipping in an iced acid brine, spoiled in approximately the same time as the untreated samples, and the pH at spoilage was 7.6 to 7.8. The bacterial counts at the time of spoilage varied widely in both acidified and untreated samples and were usually higher in the untreated samples.

Fellers (1927), in a study of the spoilage of canned crabmeat, found that a type of nongaseous spoilage was most prevalent. This spoilage was traced to the active growth of several species of "aerobic sporulation bacilli," such as *Bacillus cereus* and *B. mesentericus*. The principal changes in the meat when attacked by these organisms was a softening of texture, marked turbidity of the liquor, slight to marked abnormal odor and taste, and slight discoloration. Putrefaction was never observed in the cans of meat infected with these organisms.

EXPERIMENTAL PROCEDURE

Preparation and Examination of Sample: In our work on Louisiana crabmeat, numerous samples over a period of more than two years have been examined. The freshly picked meat was packed in snap-lock cans, then packed in ice and shipped immediately to the laboratory for examination, a distance of approximately 90 miles. After initial counts had been made (within 24 hours after leaving the packing plant) the samples were stored at a temperature of 2 to 4°C.(35.6 to 39.2°F.) and periodic counts were made until spoilage was quite advanced.

In making the counts two methods of examination were used. At the beginning of the investigation, the contents of the can were stirred with a sterile spoon and 10 grams of the sample were weighed out into a sterile

mortar and macerated with a sterile pestle. The disintegrated meat was then placed into 90 cubic centimeters of sterile distilled water, and after shaking the mixture vigorously appropriate further dilutions were made and plated out in duplicate, using nutrient agar. A number of the samples were also plated on wort agar. In the latter part of the work, 10 grams of crabmeat were weighed into a sterile, wide-mouth bottle containing broken glass, and 90 c.c. of sterile water was added to give the 1-10 dilution. This was thoroughly agitated and appropriate dilutions made from it.

Total Count and pH: The initial counts on the fresh crabmeat varied from 100,000 to 3,000,000 organisms per gram of meat, with an average of about 400,000 per gram; total counts of representative samples as determined on nutrient agar are shown (Table 1). Spoilage was usually

TABLE 1

Total Count Increases in Crabmeat on Holding at 2 to 4°C.(35.6 to 39.2°F.)

Sample No.	One-day count	Six- to eight-day count	12- to 15-day count
1	1,320,000	2,890,000	7,500,000,000
2	1,210,000	8,800,000	6,000,000,000
3	1,225,000	9,200,000	9,000,000,000
4	2,030,000	3,300,000	16,150,000,000
5	875,000	1,560,000	11,450,000,000
6	720,000	2,450,000	7,850,000,000
7	372,000	2,885,000	2,400,000,000
8	129,000	3,100,000	12,700,000,000
9	118,000	18,150,000	4,050,000,000
10	455,000	1,000,000,000	5,050,000,000

evident when the count was around 100,000,000, although considerable variation was observed with different cans of meat. Counts on wort agar were usually about 5,000 to 15,000 colonies per gram, most of which were yeast. The wort agar count varied little between fresh and decomposed samples.

It was noted that the pH of the meat varied from about 7.0 to 7.5 in fresh samples to 8.0 to 8.4 in spoiled samples, as has been previously reported. It was observed that with increase in bacterial population during holding there was also an increase in pH, but this relationship was not exact and the bacterial count could not be predicted from the pH determination. Population and pH changes in two samples of crabmeat are presented (Fig. 1). An interesting fact noted in this connection was that meat whose initial pH was experimentally lowered to about 5 spoiled at a much lower pH than the unadjusted controls.

Studies on the Flora of Crabmeat: After the total counts had been made from the nutrient agar plates, representative colonies from different plates were picked to agar slants. Owing to the occasional occurrence of a contamination, the practice was adopted of picking the colonies to peptone water, incubating at room temperature for six to 12 hours, then streaking on nutrient agar plates. Isolated colonies were then picked to agar slants. On a number of the samples subsurface colonies were picked, but since there was no difference in these and the surface colonies, this

practice was abandoned. Gram stains were made of each organism and several media inoculated for tentative classification. About 1,600 colonies were isolated and 100 of these were selected and subjected to more extensive study; the results on some of these organisms are given (Table 2). It is interesting to observe that out of over 1,600 organisms studied, not one of them was a *Proteus* species, the genus which Harris found to be most important in spoilage in his studies of Maryland and Virginia crabmeat.

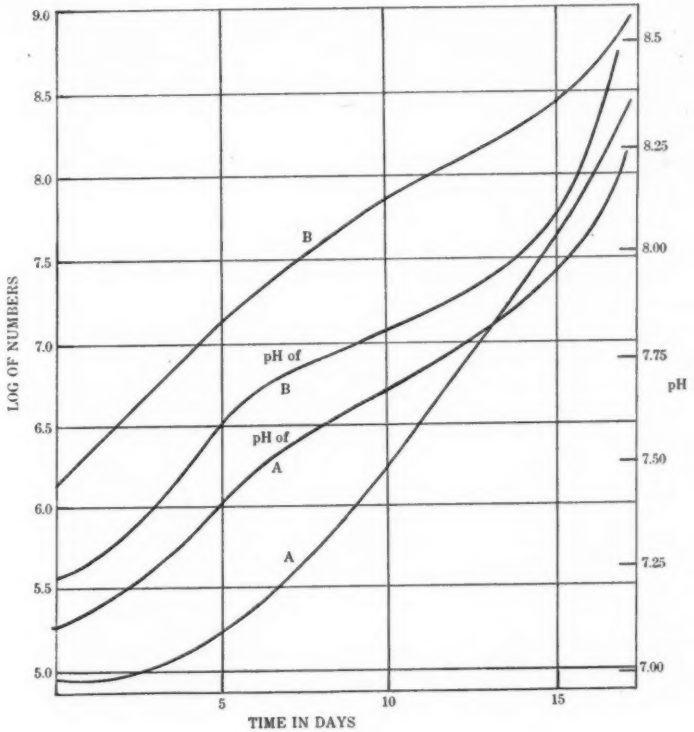

FIG. 1.

In a study of the organisms isolated from meat after various periods of holding, it was noted that there was a characteristic change in the flora as the total count increased and spoilage became evident; these observations are summarized (Table 3). In fresh samples, the flora consisted largely of cocci, but on holding there was a marked decrease in the number of cocci and a corresponding increase in *Achromobacter* and *Pseudomonas*. The flora of spoiled samples consisted almost entirely of *Achromobacter-Pseudomonas* types. Weaver (1927) found a similar change in spoiling hamburger meat.

In an investigation of the ratio of psychrophiles to non-psychrophiles in crabmeat at various stages of holding, colonies were picked directly to

TABLE 2

Characteristics of Representative Organisms Isolated From Crabmeat Stored at 1 to 4°C.(33.8 to 39.2°F.)[1]

Culture	Shape	Gram	Motility	Spores	Pigment	Dextrose	Sucrose	Lactose	Litmus milk	Nitrate red.	Indol prod.	Gelatin liq.	Tentative identification
37	Rod	—	+	—	—	A	—	—	AP	+	—	+	*Achromobacter* sp.
77	Rod	—	+	—	—	A	—	—	nc	—	—	—	*Achromobacter* sp.
368	Rod	—	+	—	—	A	A	—	nc	+	—	—	*Achromobacter* sp.
80	Rod	—	+	—	WSG	A	—	—	AP	+	—	+	*Pseudomonas* sp.
703	Rod	—	+	—	WSG	A	—	—	AP	—	—	+	*Pseudomonas* sp.
11	Sph.	+	—	—	Yel.	A	A	—	nc	+	—	—	*Micrococcus* sp.
47	Sph.	+	—	—	—	A	A	A	nc	—	—	—	*Micrococcus* sp.

[1]A = acid, P = peptonization, nc = no change, + = positive, WSG = water-soluble green pigment, Yel. = yellow pigment.

chilled agar slants and incubated at 2 to 4°C.(35.6 to 39.2°F.); the results of this work are recorded (Table 4). It can be seen that although some of the cocci isolated will grow slightly at low temperatures, these organisms either were inhibited by other organisms or did not grow fast enough to be a factor in spoilage.

Another interesting change in flora was noted in the species of *Achromobacter*. The majority of this genus picked from the fresh meat

TABLE 3

Change in Flora of Crabmeat Stored at 3 to 5°C.(37.4 to 41°F.)

Storage	Number colonies picked	Cocci	*Pseudomonas-Achromobacter*	Other bacilli
days		*pct.*	*pct.*	*pct.*
1	525	60.4	23.4	16.2
5-8	420	30.9	60.0	9.1
11-15	598	3.0	96.3	0.7

and from meat samples held only a few days fermented both dextrose and sucrose with the formation of acid. These organisms would not grow at 2 to 4°C. and disappeared in a manner similar to the cocci. Organism 368 (Table 2) is characteristic of this group. As the meat aged and decomposition became evident, the predominant types did not ferment sucrose, while most of them did ferment dextrose. These cultures grew at 2 to 4°C. and are characterized by Organism 77 (Table 2). To check further the assumption that cocci were not concerned in the spoilage of

TABLE 4

Types of Psychrophiles in Stored Crabmeat

Storage	Number isolates	Isolates which grew at 3 to 5°C.(37.4 to 41°F.)		
		Cocci	*Pseudomonas-Achromobacter*	Other bacilli
days		*pct.*	*pct.*	*pct.*
1	196	32.8[1]	15.0	36.6[1]
6	195	27.6[1]	90.0	25.0
11	199	0.0	94.0	0.0

[1] For most isolates the growth was slight.

crabmeat, pure cultures of cocci, *Achromobacter*, and *Pseudomonas* were separately inoculated into sterile crabmeat and held at 2 to 4°C. for 15 days. In no instance did the cocci produce an off-odor in the meat, whereas the *Pseudomonas* cultures produced an odor typical of spoiled crabmeat and the *Achromobacter* species produced an atypical odor. In order to determine the growth behavior of the isolates from crabmeat, selected representative strains were inoculated into nutrient broth and held at low temperatures.

The growth rate is shown (Fig. 2) of one of the typical *Pseudomonas* species in nutrient broth when held at 0°C.(32°F.) and 2 to 4°C.(35.6 to 39.2°F.). Similar curves were obtained with *Achromobacter* cultures (Figs. 3 and 4), while with a coccus species there was a steady decline

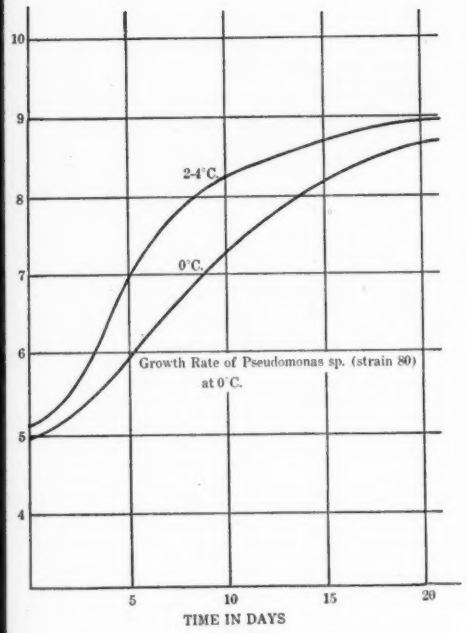

FIG. 2.

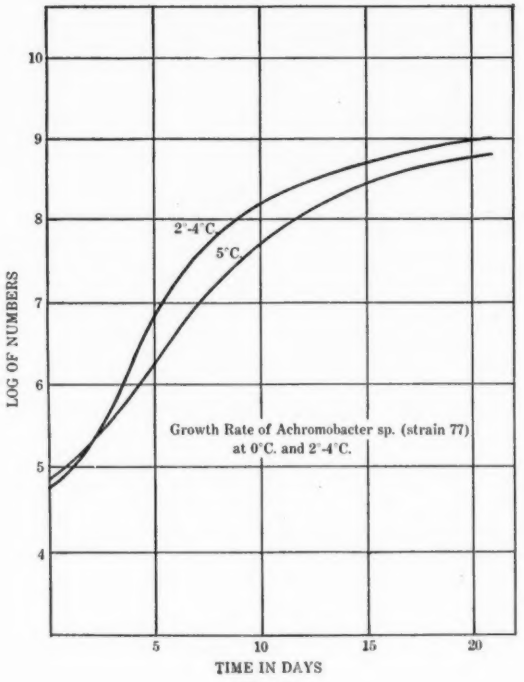

FIG. 3.

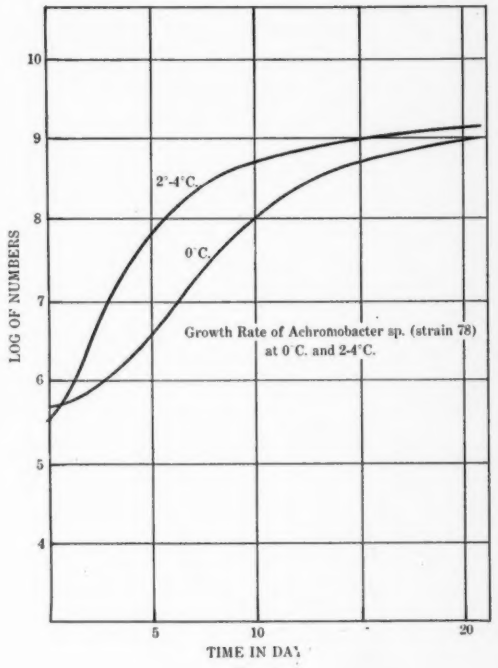

FIG. 4.

in the count. The increasing count of the *Pseudomonas* and *Achromobacter* species at these low temperatures closely follows that of the spoiling crabmeat.

SUMMARY

In this investigation of spoilage of Louisiana crabmeat, it was found that the average bacteria count of freshly packed crabmeat was approximately 400,000 organisms per gram of meat. The total counts at the time of definite spoilage varied widely but were usually around 100,000,000 per gram. Counts made on wort agar indicate that the yeast and mold count is low and remains comparatively constant throughout the period of holding. The increasing bacterial population was accompanied by an increase in pH of the meat, but an exact correlation of the two was impossible. During holding the flora of crabmeat underwent a pronounced change. Cocci, which predominated in fresh samples, were supplanted by organisms of the *Pseudomonas-Achromobacter* group which constituted about 95 per cent of the flora of spoiled samples. Although 1,600 isolates were examined, not a single organism belonging to the genus *Proteus* was encountered.

Our studies with pure cultures at temperatures of 0°C.(32°F.) and 2 to 4°C.(35.6 to 39.2°F.) indicated that the *Pseudomonas* and *Achromobacter* species are the most important organisms in the deterioration of crabmeat at low temperatures.

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SOME COOKING QUALITIES OF HOMOGENIZED MILK
I. BAKED AND SOFT CUSTARD[1]

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A number of inquiries have been received concerning the cooking qualities of homogenized milk as compared with unhomogenized or regular pasteurized milk. Although consumer preference questionnaires sent out by Tracy (1936), regarding the use of homogenized milk in the home, indicate that most women who use homogenized milk prefer it for cooking purposes, other women have expressed difficulties in its use in certain cooked products, such as cream soups, sauces, and baby formulas. Because of these differences of opinion the present study to investigate the cooking properties of homogenized milk was started.

This report deals with the use of homogenized milk in baked and soft custards. Custards were selected for the study of the cooking properties of the milk because of their high degree of sensitivity to slight changes in the egg-milk-sugar mix. This sensitivity of custards to changes in the mix was demonstrated in a recent report on the cooking quality of four grades of eggs by Logue (1940). In this the firmness of the gel formation of the custards showed a direct relationship to the grade of eggs. The eggs which graded higher as scored by the standard methods of grading and the Haugh Units, gave a firmer coagulum in both the baked and soft custard than did the eggs that graded lower. Since Logue's results indicate that custards are a successful measure of determining the cooking quality of the grade of eggs, it seemed to the authors that custards should also be a valuable medium for studying the cooking properties of homogenized milk.

With the exception of a brief report of the work done by Hollender and Weckel (1940) at the University of Wisconsin, little has been published on the effect of homogenization of milk on its cooking properties. The work done at the University of Wisconsin indicates that in certain types of cookery the homogenized milk curdles more easily than other milk. This was true especially when homogenized milk was used in cooking oatmeal or wheat cereals. Extremely hard water favored curdling. A lack of salt in cereals, however, favored curdling, although the addition of some salt tended to prevent it in the cooked product. When homogenized milk was used in egg custards, it was found that less serum separated when the material was cooked at a low temperature for a short time. Homogenized milk in escalloped potatoes showed a strong tendency to curdle. The extent of curdling was found to depend on the quality and chemical composition of the potato. The addition of salts, such as

[1]Approved by the director of the Michigan Agricultural Experiment Station for publication as Journal Article No. 567, N. S.

sodium citrate, sodium carbonate, and sodium phosphate, did not prove to be a reliable means of preventing curdling of the milk in escalloped potatoes.

Research on the physical, chemical, and organoleptic properties of homogenized milk has been done by a number of workers interested in dairy science. The relationship between the changes of the physical and chemical properties of milk owing to homogenization and its cooking properties are not well defined. However, the differences in the cooking properties brought about by homogenization may be studied either directly or indirectly in the light of these physical changes. Among other changes in milk owing to homogenization is the decrease in the size of fat globules resulting in their complete dispersion. Probably of equal or even greater importance in cookery, however, is the decrease in protein stability of milk toward alcohol as a result of homogenization. Also, the curd tension of milk as measured by the Hill Test is markedly lowered by the process.

EXPERIMENTAL PROCEDURE

Both the baked and soft (stirred) custards were made from whole pasteurized milk, homogenized and unhomogenized, obtained fresh each day from the College creamery. The eggs used in making the custards were also fresh, since Logue (1940) found that the age of the egg influenced the quality of the custard. The white and yolk from a number of eggs were weighed in definite proportions, and mixed with a rotary beater. Care was taken to mix the white and yolk thoroughly but not bring about partial coagulation of the protein as that would later affect the quality of the custard. The egg used in each batch of custard made with homogenized and unhomogenized milk was taken from this common supply. Thus, the eggs used in each day's series were homogeneous.

The proportion of ingredients in the baked and soft custards was the same, consisting of 61 grams of egg white, 35 grams of egg yolk, 488 grams of milk, and 40 grams of sugar. This is roughly equivalent to two whole eggs, two cups of milk, and three and one-half tablespoons of sugar. The method of cooking the two types of custard was different. The baked custards were cooked in an oven without stirring, while the soft custards were cooked in a double boiler and stirred continually. Those not stirred were firmer in texture and appeared to be in one piece or clot; those stirred had a softer texture and were viscous fluid.

The method of preparing the custard mix was as follows: The milk, especially the unhomogenized, was mixed thoroughly to insure even distribution of the cream throughout the bottle. The milk was then weighed out in a double boiler; that which was used in the baked custards was heated to 50°C.(122°F.) before the sugar was added. This was done in order to obtain complete solution of the sugar before aliquot portions were taken for the individual sample. After the eggs and sugar were thoroughly blended with the milk, the mix was strained to remove the chalazae, after which the individual samples were weighed out. Four 145-gram samples of the mix were weighed into standard-size, pyrex custard cups (6½ cm. wide at top, 4 cm. at bottom, and 5½ cm. deep) to be used

for the baked custards. The cups were then placed in a deep baking pan; water at 30°C.(86°F.) ± two degrees C. was then poured into the pan until it came up to the level of the custard mix. The cups containing the custard made with homogenized and unhomogenized milk were arranged alternately in the same baking pan. The pan was placed on the lower rack of an oven regulated at 162.8°C.(325°F.). Thermometers were suspended from a rack in the top of the oven into the center of the custard mix. Temperature readings were taken every 15 minutes during the first 45 minutes that the custards were in the oven and every five minutes thereafter until the interior temperature of the custards had reached 86°C.(186.8°F.). The custards were then removed from the oven and allowed to stand at room temperature before subsequent tests were made.

The soft custards were cooked by placing the mix in the top of a double boiler. Stirring was continued throughout the cooking period. Since maximum coagulation of the protein in custard is a factor of time of cooking as well as temperature, the rate of heating was carefully regulated. An electric plate which had three speeds, low, medium, and high, was used to cook the custards in order to control carefully the amount of heat throughout the cooking period. The top of the double boiler containing the custard mix was placed in the bottom of the boiler which contained approximately one quart of water at 25°C.(77°F.). A thermometer was suspended in the mix and adjusted to clear the bottom of the pan one-sixteenth of an inch. The electric plate was preheated on high speed before the double boiler was placed on it. The high speed was maintained until the custard reached 70°C.(158°F.). Low speed was then used until the interior temperature reached 83°C.(181.4°F.) at which time it was poured immediately into four standard pyrex custard cups and allowed to stand undisturbed for five hours before testing. The temperature during cooking was recorded every five minutes for the first 15 minutes and every minute thereafter. The time required to cook each batch of custard was 37 ± two minutes.

Objective and subjective tests were used to compare the baked and soft custards made from homogenized and unhomogenized milk. The objective tests used for the baked custards consisted of rate of heat penetration by means of time-temperature scale, standing index, amount of syneresis on standing, and firmness of custard as measured by the Curd Tension Meter. The objective tests on the soft custards consisted of viscosity measurements as determined by the MacMichael viscosimeter. The judges' score sheets for the baked and soft custards included the factors of sweetness, texture, color, and firmness.

The rate of heat penetration was studied by recording the initial temperature of the baked custards and the temperature every 15 minutes for the first 45 minutes and at five-minute intervals throughout the rest of the cooking period.

The standing index was determined on the baked custards two hours after they were removed from the oven and again at the end of five hours. This was accomplished by removing the custards from the cups onto a flat surface. Measurements of height and width were taken immediately after removal from the cup and at the end of three hours of standing at

room temperature. The standing index is the ratio of the height of the custard to the average diameter when measured at the widest part.

The amount of syneresis was determined by weighing the liquid that separated out after the custards had been removed from the cups and allowed to stand at room temperature for three hours.

The firmness of the custard was measured by the Curd Tension Meter illustrated (Fig. 2). This instrument consists of a cutter and a float assembly which floats freely in a pool of mercury that is carried in a glass tube secured to a rack arrangement. This rack was moved down at a constant speed by a small motor. By removing the baked custard from the cup and placing it inverted in the path of the downward moving cutter, the cutter is brought in contact with it and the amount by which the float is raised out of the mercury is a measure of the resistance offered to the passage of the cutter through the custards. thus providing a measure of the firmness of the coagulum. The custards were allowed to stand five hours before this testing. They were, however, tested immediately upon removal from the cup.

Viscosity measurements were determined on the soft custards by means of the MacMichael Viscosimeter using standardized No. 26 gauge wires, the bowl traveling at 20 R.P.M. These tests were also made five hours after the custards were cooked. Reading on the viscosimeter was taken at the highest point, which was reached approximately 15 seconds after the motor was started, and at the end of every minute for five minutes.

The baked and soft custards were judged subjectively by a group of staff members at Michigan State College who were experienced in scoring dairy products.

DISCUSSION OF RESULTS

Heat Penetration and Cooking Time: The results of a large number of time-temperature readings made to determine the rate of heat penetration in baked custards prepared with the two kinds of milk (Table 1 and Fig. 1) indicate that the rate of heat penetration in custards made with homogenized milk is slower by 15 to 20 minutes than in custards made with unhomogenized milk. Thus the cooking time is increased approximately 15 minutes for custards made with homogenized milk. Preliminary tests showed that custards made with homogenized milk could withstand higher baking temperatures than custards made with plain milk without seriously affecting the stability of the gel. Of course, when a higher baking temperature is used the baking time is reduced.

Standing Index: The standing index, which is a ratio of height of the custard to the average diameter, was used as a measure of quality of the custard or its ability to hold its shape when removed from the cup and inverted on a flat surface. When the gel structure was weak the custard slumped. Therefore the width increased and the height decreased, thus reducing the standing-index value. Since the size of the cups and the amount of mix in each cup was the same, the results among the custards were comparable.

The average standing-index value of the baked custards made with homogenized and unhomogenized milk is shown (Table 2). The differences

were tested statistically according to Fischer's (1936) "t" test for significance. This test showed that homogenized milk gave custards with significantly higher index values than unhomogenized milk both when measured immediately after the custards were removed from the cups

TABLE 1

Rate of Heat Penetration in Baked Custards Prepared With Unhomogenized and With Homogenized Milk

(Average of eight trials)

Time of heating	Internal temperatures of baked custards at specific time intervals when prepared with milk	
	Unhomogenized	Homogenized
min.	*°C.*	*°C.*
0	33.6	33.4
15	49.4	48.3
30	63.8	62.8
45	74.8	72.5
50	78.3	75.9
55	80.5	77.9
60	82.2	79.1
65	83.9	80.9
70	85.1	82.0
75	86.0	83.1
80	.....	84.0
85	.....	85.3
90	.....	86.0

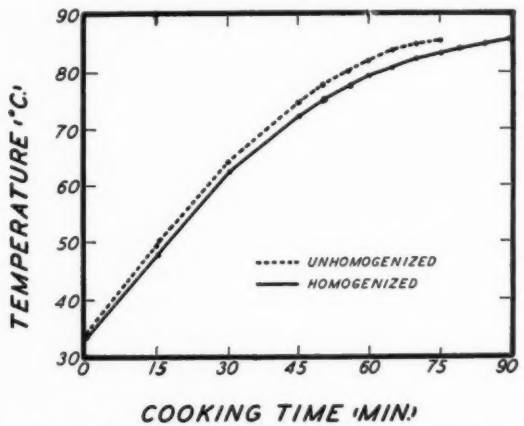

FIG. 1. Rate of heat penetration in baked custards made with homogenized and unhomogenized milk.

and at the end of three hours, thus indicating that the homogenized milk produced a better gel formation in custards than unhomogenized milk.

Stability of Gel: The effect of homogenization of milk on the stability of the gel in baked custards was measured by the amount of syneresis, or separation of the liquid and the coagulum into two phases. Tendencies to

syneresis may occur to a greater or lesser extent depending on the quality of the gel formation. Nason (1939) says the formation of egg gel, such as in baked custards, depends upon the coagulation of the protein and the ability of the precipitated protein to hold within its meshes the solution from which it was precipitated.

The average amount of liquid separating from baked custards made with the homogenized milk was 1.51 grams, with the unhomogenized milk 5.57 grams. (Experiment included 12 trials, six replicates each.) Apparently the quality of the gel is better in custards made with homogenized than those made with unhomogenized milk since there is less syneresis upon standing in the former. The differences in the amount of syneresis between custards made with homogenized and unhomogenized milk were

TABLE 2

Standing Index of Baked Custards

Testing time after removal from oven	Standing index of baked custards prepared from milk		"t" value of difference
	Homogenized	Unhomogenized	
At end of 2 hr.[1]	.558	.512	4.68[3]
At end of 5 hr.[2]	.475	.422	7.23[3]

[1] Eleven trials, six replicates each. [2] Fourteen trials, six replicates each. [3] Significant within one per cent limit.

statistically significant within the one-per cent level, according to Fisher's "t" test ($t = 3.72$).

Curd Tension of Custards: Another measure of the differences in gel formation in baked custards made with homogenized and unhomogenized milk was that of firmness or resistance to the cutting blades of the Curd Tension Meter, commonly referred to as curd tension. This test of firmness of the custard was carried out using the instrument shown (Fig. 2).

With the proportion of egg to milk used in this formula, as firm a custard as possible was considered most desirable. A series of readings was taken on custards made with the two types of milk and the results were compared. Custards made with homogenized milk gave a firmer coagulum than those made with unhomogenized milk, according to the Curd Tension Meter readings. The average curd tension on custard made from homogenized milk was 19.2 grams as compared with an average of 13.26 grams on those prepared with unhomogenized milk (the average of 15 trials, five replicates each). This difference in firmness of the custards was shown by Fischer's "t" test to be highly significant.

Color and Appearance of Baked Custards: The color and consistency of the top of the baked custards made with the two types of milk were quite different. In every case the custards made with the unhomogenized milk formed a soft crust with an even, brown color on top, while the custards made with homogenized milk formed a tough skin and browned very little on top except at the extreme edges of the cup. Evidently, this difference was due to the butterfat which collected at the top of the unhomogenized custard mix because when a small amount of butter or cream

was placed in the cups containing the homogenized custard mix they too browned and formed a softer crust on top.

Viscosity of Soft Custards: Since the texture of the soft custard was that of a viscous fluid, the previous tests, applicable to measure the gel formation of baked custards, were not satisfactory for soft custards. The comparative viscosities of the soft custards from homogenized and unhomogenized milk were measured by the MacMichael viscosimeter. As

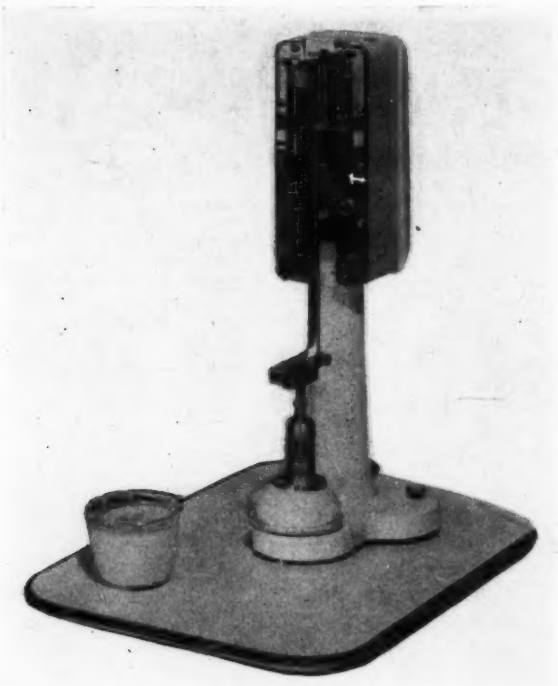

FIG. 2. Determining the curd tension of baked custards by means of the Curd Tension Meter.

previously indicated, readings were taken on the custards after they had reached the highest point, which required approximately 15 seconds, and at the end of each minute for five minutes. The average readings of 10 experiments with four replicates in each experiment are presented (Table 3 and Fig. 3). The curves in the figure show clearly the difference in viscosity of custards made with homogenized and unhomogenized milk, the homogenized milk yielding a more viscous custard. Both curves show a gradual parallel decline in the viscosity during the testing period owing to a breaking down of the gel structure. Fisher's "t" test for difference was applied at the one-minute and five-minute periods. The results of these

tests show the differences in viscosity at the two periods of testing to be highly significant.

JUDGES' PREFERENCE OF CUSTARDS

The effect of homogenization of milk on the palatability of baked and soft custards was studied by having 16 judges who were experienced in scoring dairy products register their preferences on several quality items

TABLE 3

Viscosity Readings of Soft Custards Made With Homogenized and Unhomogenized Milk

(Average 10 experiments, four replicates each)

Type of milk	Highest reading	Reading at end of (minutes)				
		1[1]	2	3	4	5[2]
Homogenized	352.4	296.0	276.3	262.5	251.2	242.5
Unhomogenized	290.7	229.3	214.5	205.1	197.1	191.4

[1] "t" value at one minute 5.16. [2] "t" value at five minutes 5.07.

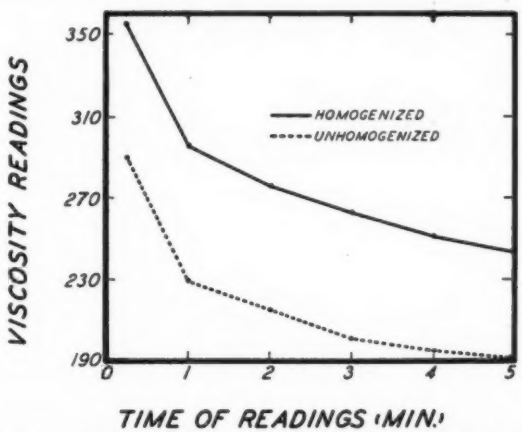

FIG. 3. Viscosity of soft custards made with homogenized and unhomogenized milk.

of paired samples. The baked custards were turned out of the custard cups onto paper plates before judging so the judges would not be influenced by the surface appearance. Information relative to the custards was withheld from the judges until after they had stated their preferences; the distributions of the judges opinions are included (Table 4).

Fifteen of the 16 judges scored custards made with homogenized milk as firmer in consistency than custards made with the normal pasteurized whole milk. These results coincide closely with the results on the standing index and with the Curd Tension Meter for firmness of the custards.

There was indication of a slight preference for the color of the custards made with homogenized milk; however, six of the 16 judges had no preference at all with regard to color. Although both custards were desirable in color it should be mentioned that those made with homogenized milk were always slightly lighter in color than those made with unhomogenized milk.

An interesting item which was brought out in the judges' opinions and one which follows closely some of the other findings in the investigation was that of sweetness of the baked custards. To the majority of judges, the custards made with unhomogenized milk appeared to be sweeter in flavor than the custards made with homogenized milk. Inasmuch as custards made with unhomogenized milk showed a greater tendency toward syneresis than those made with homogenized milk, it is very likely the sugars are concentrated in the liquid phase and, therefore, appear to be sweeter to the taste although actually the same amount of sugar was present in each custard. A general comment made by a number of observers

TABLE 4

Distribution of Judges' Opinions on Certain Qualities of Baked and Soft Custards Made From Normal Pasteurized Whole Milk and From Similar Milk Homogenized

Factor observed	Distribution of preferences			
	Baked custard			
	Unhomogenized		Homogenized	
	Number	Per cent	Number	Per cent
Color[1]	4	25.00	6	37.50
Firmness	1	6.25	15	93.75
Smoothness	8	50.00	8	50.00
Sweetness[2]	9	56.25	5	31.25
General preference	8	50.00	8	50.00
	Soft custard			
General preference	1	6.25	15	93.75

[1] Six of the 16 judges had no preference. [2] Two of the 16 judges had no preference.

Comments: Baked custards—"No. 1 (homogenized) is more like custard as to consistency, No. 2 is more like cornstarch pudding." Soft custards—"No. 1 (homogenized) has heavier body, No. 2 loses its sweetness too rapidly."

who tasted the two types of custard was that in custards made with homogenized milk the flavors of the egg, sugar, and milk were better blended.

The judges showed no general preference for baked custards made with unhomogenized milk over those made with homogenized milk or vice versa, although they did express a preference concerning some factors of the custards. However, it must be considered that the samples of custards judged in this experiment were small in quantity, and here the firmness of the custard would not be as important to the quality of the product as in the case of custard pie.

SUMMARY AND CONCLUSION

The effect of homogenization of milk on some of its cooking properties was studied by means of baked and soft custards. The cooking time for custards made with homogenized milk was found to be from 15 to 20 minutes longer than for custards made with similar unhomogenized milk because the rate of heat penetration was more rapid in the latter.

Baked custards made with homogenized milk gave significantly higher standing-index values both when measured immediately after removal from the cup and when measured after standing for three hours at room

temperature than those made with unhomogenized milk. This would indicate better gel formation in the former. The firmness of the custards was measured both objectively by means of a Curd Tension Meter and subjectively by means of the judges' opinion. A close correlation existed between the two methods inasmuch as the custards made with homogenized milk were rated firmer in both cases.

The amount of syneresis upon standing for custards made with homogenized milk was significantly less than for custards made with unhomogenized milk.

Soft custards made with homogenized milk gave a more viscous custard than those made with unhomogenized milk, according to the MacMichael viscosimeter.

The judges' opinion on the palatability of the custards made with the two types of milk showed no preference in the baked custards, but a decided preference for soft custards made with homogenized milk.

It is concluded that both baked and soft custards made with homogenized milk are superior in quality to those made with unhomogenized milk.

Since this study was completed and the manuscript written the paper "Stability of Homogenized Milk in Cookery Practice" by Hollender and Weckel has appeared in FOOD RESEARCH 6, 335-343. The data presented in the present paper does not substantiate their findings. However, the conditions of the experiments were quite different.

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ASCORBIC ACID CONTENT OF COW'S MILK AFTER FIVE YEARS OF CONTINUOUS LACTATION

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During an extended investigation of factors which may influence the ascorbic acid content of cow's milk, an opportunity developed for studying the ascorbic acid content of milk from a cow which had been in continuous production for over five years. The cow was a grade Holstein, 11 years old, and weighed 1,300 pounds. For six years or more previous to this study she had been fed the rations and maintained under the conditions described in previous publications by Holmes *et al.* (1939a, 1939b, 1940a, 1940b). This cow was mated at frequent intervals during the five-year lactation period but in each instance she failed to conceive. However, she was retained as part of the certified milk herd for even after this unusually long lactation period she was producing eight liters of milk per day.

In this study the first sample of milk assayed for its ascorbic acid content was collected during the 62nd month of lactation. The samples of milk were collected at eight bimonthly intervals from the morning milking at 3:00 A.M. They were rapidly cooled and stored out of contact with light until they were assayed. The ascorbic acid content of the milk was determined by the indophenol titration method (1939a). The results of the assays of the milk which include data for the month of lactation, the amount of milk produced, the ascorbic acid per liter of milk, and the total daily production of ascorbic acid are reported (Table 1).

TABLE 1

Ascorbic Acid Content of Cow's Milk

Month of lactation	Milk production daily	Ascorbic acid per liter	Daily ascorbic acid production
	lit.	*mg.*	*mg.*
62	8.01	24.05	192.6
63	8.01	23.22	185.8
63	7.97	26.02	207.3
64	7.97	33.54	267.3
64	7.66	24.63	188.8
64	8.23	23.48	193.3
65	8.37	24.06	201.3
65	8.32	20.34	169.2
Average	8.07	24.91	200.7

The ascorbic acid content of the milk varied from 20.34 to 33.54 mg. and averaged 24.91 mg. per liter during the approximate four months' experimental period. For some unexplained reason the ascorbic acid content of the fourth sample of milk was unusually high, 33.54 mg. per liter. The volume of milk produced remained practically constant, varying only from 7.66 to 8.37 liters per day with an average of 8.07 liters. The total amount of ascorbic acid secreted daily in the milk produced by the cow used in this study varied from 169.2 to 267.3 mg. and averaged 200.7 mg. for the experimental period. Since the amount of milk obtained was

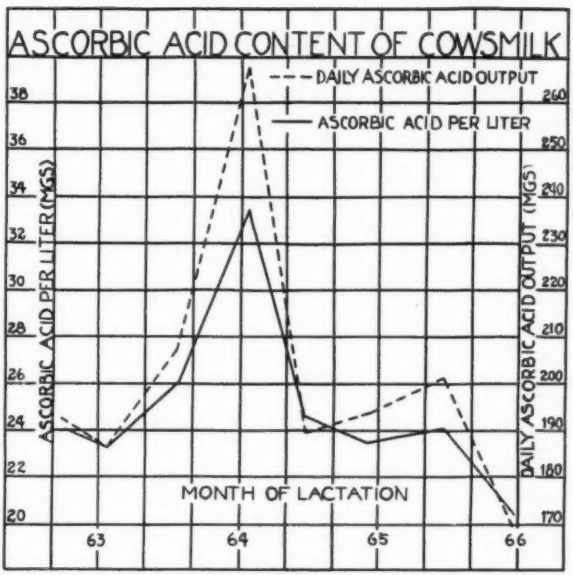

FIG. 1.

nearly constant, the amount of ascorbic acid produced daily varied with the ascorbic acid content of the milk. The ascorbic acid content per liter of milk and the total daily ascorbic acid output from the 62nd through the 65th month of lactation are shown (Fig. 1).

It is interesting to note that this Holstein cow, after a continuous lactation period exceeding five years, produced milk with an ascorbic acid content (24.91 mg. per liter) greater than the average ascorbic acid content (18.17 mg. per liter) of 337 samples of milk (1939a) produced by a herd of Holstein cows which received the same rations and were kept under the same housing and management conditions. Thus it appears that the Holstein cow under discussion established a most unusual record.

The authors wish to acknowledge the very helpful assistance of Mr. A. A. Colley and Mr. E. C. Steele during the course of this study.

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DIGESTION OF BEEF BY PAPAIN[1]

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The plant proteinase, papain, has found considerable application as a meat tenderizer but its use for this purpose has been largely empirical. The digestion of whole beef muscle and the survival of papain heated with whole muscle have not been studied quantitatively.

The procedure usually recommended for tenderizing meat with papain is to paint the meat with a solution of the enzyme, then cook it after an interval of a few minutes. To be of use, the enzyme action must be rapid. Obviously, the proteolytic action under such circumstances depends upon such factors as the optimum temperature for the digestion of meat by papain, the stability of papain at cooking temperatures in the presence of meat, the extent of contact between the enzyme and the meat, the ability of papain to penetrate the meat during digestion, and the digestibility of the native compared with the heat-denatured proteins. In the experiments here described an attempt has been made to estimate the importance of several of these factors. The results obtained are outlined in the summary.

The effect of tenderization in meat is difficult to treat quantitatively because adequate criteria of what constitutes tenderness are lacking. The tenderness induced by proteolytic enzymes, however, is a result of protein breakdown. Although the exact relationship between tenderizing effect and protein digestion is not known, it was apparent throughout this work that, in general, the more the meat was digested as determined chemically, the softer the structure of the meat became. The digestion of meat by papain was followed by measuring the blue color which the nonprotein digestion products give with the Folin-Ciocalteau phenol reagent after precipitation of the proteins with trichloracetic acid. This color is due to tyrosine, tryptophane, and cysteine and is expressed in terms of the amount of tyrosine which would give the same color, Anson (1938). The survival of papain heated with meat under various conditions was followed by the hemoglobin method for measuring papain activity.

Our results (Table 1) show that 80°C.(176°F.) was the most favorable temperature for the rapid digestion of ground meat by papain during a 30-minute period. From 70 to 85°C.(158 to 185°F.), however, the amount of digestion by papain did not vary much. Over this temperature range during the 30-minute digestion period, the increase in reaction rate brought about by raising the temperature was, presumably, compensated for by inactivation of the enzyme. Above 85°C., raising the temperature

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[2] Enzyme Research Laboratory Contribution No. 76.

had more effect on the denaturation and inactivation of the enzyme than on increasing the rate of digestion. The optimum temperature for the digestion of meat and also pure proteins by papain is higher than the optimum temperature for the activity of most other enzymes because papain withstands a higher temperature than most enzymes without being inactivated. Our experiment (Fig. 1) was carried out for a 30-minute period. In general, with enzymes, the optimum temperature for maximum digestion is lower the longer the period of digestion, according to Northrop (1939). Therefore, beef heated with papain for longer than 30 minutes should probably be heated at a temperature below 80°C.

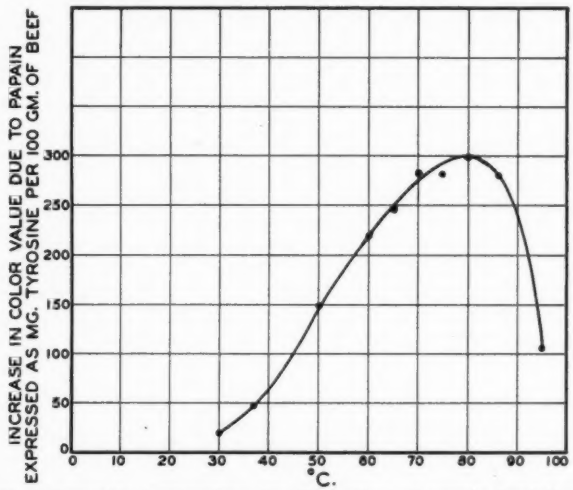

FIG. 1. Most favorable temperature for the action of papain heated with ground beef for 30 minutes (.3 mg. papain paste added for each gram of beef).

During a four-hour digestion period papain split off about the same amount of tyrosine from blocks of beef at 75 and 55°C.(167 and 131°F.). At the higher temperature, during four hours, enzyme inactivation also appears to have compensated for any tendency toward increase in reaction rate.

The rate of papain digestion at 55°C. is shown (Fig. 2). Blocks of unground meat were digested most rapidly during the first four hours and large amounts of tyrosine were liberated. The beef was very much softened [3] within four hours and disintegrated within 28 hours. At the end of 28 hours none of the papain had been destroyed (Table 2).

The results of papain digestion at room temperature and at the cooking temperatures ordinarily employed were very inferior to those obtained at 55 and 75°C. Papain did not appreciably digest blocks of beef at room

[3] This softening, brought about by three mg. of papain paste per gram of meat, was much greater than would be desirable in cooking meat. This paper describes the conditions influencing papain digestion and not the precise conditions for its use in tenderizing.

temperature (Table 1). This result can be explained by our experiments with beefsteak (Table 3), which showed that at 23°C.(73.4°F.) in 20 minutes, papain was unable to penetrate farther than 2.5 mm. below the surface of the undigested beef, and probably did not penetrate that surface at all. There is therefore, no advantage in pretreating meat with papain before cooking as any digestion caused by the enzyme takes place during the cooking process.

Blocks of beef immersed in papain and then heated at 100°C.(212°F.) in boiling water or baked in an air oven at 155°C.(311°F.) were only

TABLE 1

Digestion of Ground and Unground Beef Treated With Papain at Room Temperature and at Ordinary Cooking Temperatures

Beef sample	Method of cooking after immersion in papain solution for 5 minutes at room temperature	Increase in color value owing to digestion (mg. tyrosine per 100 gm. beef)
Blocks, raw	None	11
Blocks, previously boiled 5 min.	None	20
Ground beef, raw	None	81
Ground beef, previously boiled 5 min.	None	89
Blocks, raw	None	4
Blocks, baked 25 min.	None	1
Ground beef, raw	None	60
Blocks, raw	10 min. in boiling H_2O	63
Ground beef, raw	10 min. in boiling H_2O	522
Blocks, raw	10 min. in boiling H_2O	51
Blocks, boiled 5 min.	10 min. in boiling H_2O	33
Blocks, raw	25 min. in oven at 155°C., uncovered	71
Blocks, baked 5 min.	25 min. in oven at 155°C., uncovered	65
Ground beef, raw	25 min. in oven at 155°C., uncovered	530
Blocks, raw	25 min. in oven at 155°C., uncovered	193
Blocks, baked 10 min.	25 min. in oven at 155°C., uncovered	128
Ground beef, raw	25 min. in oven at 155°C., uncovered	373

slightly softened, and the amount of tyrosine liberated (Table 1) was not nearly so great as that liberated at 55°C. (Fig. 2) or 75°C. Other things being equal the extent of digestion during heating depends on the ability of the enzyme to survive the cooking temperature and to penetrate the meat. In the course of a few minutes' heating with beef at 100°C., the destruction of papain was complete (Table 2), whereas at 55°C. the papain has been shown to be stable for several hours. The extent of papain digestion in a 155°C.(311°F.) oven depends, therefore, on whether the enzyme is on the inside of the meat where the temperature (65 to 75°C.) is favorable to papain action, or on the outside of the meat where the papain is readily destroyed. When the outer layer of the meat is softened by digestion at a favorable temperature the enzyme "eats its way" into the interior of the meat by continuous liquefaction and digestion of the exterior layers.

Under all the conditions studied ground beef was digested and softened very much more rapidly than unground beef. Although blocks of beef

were not measurably affected by papain at room temperature in a few minutes, it can be seen (Table 1) that ground beef was digested. At 55°C., more tyrosine was liberated from ground beef (Fig. 1) than was split off by 10 times as much papain from unground meat (Fig. 2). At 100 and 155°C. (Table 1) there was also considerably more digestion of ground than unground meat. During the time the ground meat, while being heated at the usual cooking temperatures, was passing through the optimal temperature range for digestion by papain, more meat was in contact with the enzyme, and the enzyme in the interior of the ground meat did not reach the inactivation temperature as quickly as the enzyme on the outside layer of the blocks of beefsteak.

It is noteworthy that, as is also shown (Table 1), the previous cooking of either ground or unground meat did not increase the rate of digestion by papain at room temperature or at higher temperatures.

TABLE 2

Survival of Papain After Cooking With Unground Meat

Method of cooking	Time of cooking	Papain found after cooking (Hb. units per gm. beef $\times 10^{-2}$)
	min.	
Water, 100°C.(212°F.)	0	1.4
	5	0
	10	0
	hr.	
Oven, 55°C.(131°F.), in covered vessel	0	1.3
	0.5	1.4
	2	1.6
	4	1.6
	28	1.6

The experiments just discussed imply certain practical considerations in the use of papain as a tenderizing agent. To be effective, the enzyme must be brought into the interior of the meat by one device or another. The temperature of the meat should be maintained below 80°C.(176°F.) during the time that the enzyme should function. Thereafter, the product must be used shortly, or special steps taken to destroy the remaining enzyme.[4]

EXPERIMENTAL PROCEDURE

The beef muscle was lean round steak cut from the carcass eight to 10 days after slaughter. The unground meat referred to throughout the paper consisted of rectangular blocks weighing 20 grams and measuring approximately 2 x 3 x 3 cm. For comparison 10-gram samples of ground meat were used with proportionate amounts of enzyme. The ground meat was prepared by extruding through a grinder plate with $\frac{3}{16}$-inch openings.

The papain employed was a paste of papaya latex in sodium chloride solution, according to Balls, Lineweaver, and Schwimmer (1941), contain-

[4] The results of digesting meat with pineapple juice paralleled those obtained with papain except that the optimum temperature for a 20-minute digestion period was about 60°C.(140°F.).

ing about 55 per cent total solids. One cubic centimeter of solution which contained four milligrams of the various paste preparations clotted 10 c.c. of buffered milk at 40°C.(104°F.), Balls and Hoover (1937), in 35 to 65 seconds without additional activator. About 90 per cent of the papain in the paste was in the activated form.

Throughout the series of tests the preliminary treatment of the meat and the temperature was varied, and the amount of proteolysis occasioned by papain was determined.

Estimation of Digestion Products: Digestion was followed by precipitating the meat proteins with trichloracetic acid and estimating the

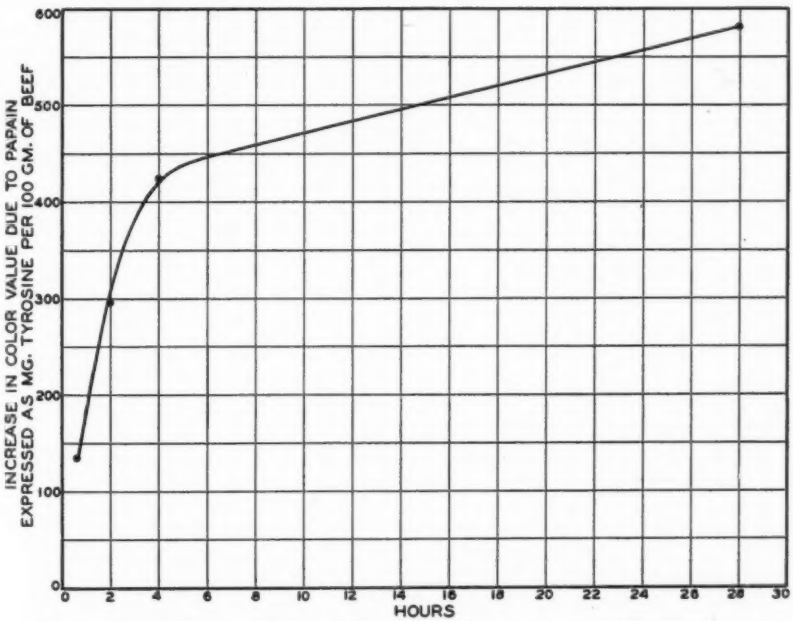

FIG. 2. Digestion of unground beef heated with papain at 55°C.(131°F.) (three mg. papain paste added for each gram of beef).

unprecipitated digestion products present in the trichloracetic acid filtrates by the blue color which they give with the Folin-Ciocalteau phenol reagent according to the directions of Anson (1938).

Protein-free filtrates of untreated and enzyme-treated beef were prepared by adding to the meat sufficient trichloracetic acid to give a volume of 10 c.c. per gram of meat and a final concentration of *.2M* trichloracetic acid in the filtrate. In the case of untreated beef 67 c.c. of *.6M* trichloracetic acid and 113 c.c. of water were added to 20 grams of beef. In the case of treated beef care was taken that all the liquid used and any juice formed during the test were included in the final volume. The mixtures

were homogenized in a mechanical beater for about three minutes, and then filtered through Whatman filter paper No. 42.[5]

The color reaction with the protein digestion products was carried out on the trichloracetic acid filtrates. In the case of enzyme-treated samples, two c.c. of the trichloracetic acid filtrates were diluted to 50 c.c. with *.2M* trichloracetic acid, and five c.c. of the diluted filtrates were used for each determination. With untreated beef 10 c.c. of the filtrate were diluted to 50 c.c. with *.2M* trichloracetic acid, and five c.c. of this diluted filtrate were used for each estimation. The color was developed by adding 10 c.c. of *.5N* NaOH to five c.c. of the diluted trichloracetic acid filtrates placed in 50-c.c. Erlenmeyer flasks. Three c.c. of the diluted phenol reagent (one volume diluted with two volumes of water) were then added dropwise

TABLE 3

Penetration of Papain Into Unground Beef Muscle at 23°C.(73.4°F.)

Slice of beef	Thickness of slice	Color value after incubation (mg. tyrosine per 100 gm. meat)
From Sample 1, 2-2.5 cm. thick	*mm.*	
1. Bottom[1]	5-10	161
2.	5-8	75
3.	10	70
4. Top	10-15	75
From Sample 2, 4-5 cm. thick		
1. Bottom[1]	2.5	351
2.	2.5	92
3.	2.5	85
4. Top	2.5	91
Control—untreated beef		87
Control—beef, but no papain, placed in .02M NaCN overnight		82

[1] This surface was dipped in the enzyme solution.

while the flask was carefully whirled. The colored solutions were transferred to calibrated tubes and the color intensity measured in an Evelyn photoelectric colorimeter, using Filter No. 635. The galvanometer of the instrument was set with colorimeter blanks prepared by adding the color reagents to five c.c. of *.2M* trichloracetic acid.

Determinations were made of the color value of filtrates of papain-digested beef. Blank determinations were also carried out on filtrates of beef not digested with papain, to which papain was added after trichloracetic acid. The difference between the color values of these two filtrates represented the increase caused by papain digestion.

The color value for untreated beef was usually 43 to 62 mg. tyrosine per 100 grams of beef; in only four cases was it higher (70 to 91 mg.).

[5] When meat was treated with papain for long periods at elevated temperatures some of the meat proteins were made insoluble by heat and others broken down by digestion. When the insoluble fraction was filtered off, the filtrate formed no further precipitate with trichloracetic acid. The acid was thus unnecessary as a protein precipitant with such samples, but was nevertheless added in order to inactivate any remaining papain and to maintain uniform analytical conditions throughout the series.

Heating beef in the absence of papain did not increase the color value.

Addition of papain (20 mg. per gram of beef) after trichloracetic acid increased the color value of the beef filtrate about seven mg. per 100 grams of beef. Heating papain increased its color value, so that smaller amounts of papain were added to beef digested at raised temperatures. Papain (three mg. for each gram of beef), heated at 55°C.(131°F.) for two hours or 20 hours, and then added to beef after trichloracetic acid, increased the color value of the beef filtrate only 22 mg. In all experiments therefore, the color value attributable to papain (which in any event was always included in the beef and papain blank) was an insignificant fraction of the increase in color value resulting from papain digestion.

Optimum Temperature (Fig. 1): These experiments were made on ground beefsteak because it affords a uniform and reproducible substrate. Three c.c. of papain paste solution containing one mg. of the paste per cubic centimeter were added to 10-gram samples of beef and the mixture was brought to the desired temperature in a water bath. Stirring was continued throughout the 30-minute digestion period; the mixture was then cooled in ice water, trichloracetic acid added, and the tyrosine color value determined.

Digestion of Unground Beef at 55°C. (Fig. 2): Twenty-gram blocks of beef were heated in a 55°C. oven (in covered weighing bottles to prevent drying) with three c.c. of papain paste solution containing 20 mg. of paste per cubic centimeter. After heating, the weighing bottles and their contents were cooled in ice water, trichloracetic acid was added, and the tyrosine color value determined.

The same procedure was followed when heating beef with papain at 75°C.(167°F.). Results expressed as increase in color value (mg. tyrosine per 100 grams of beef) during a four-hour digestion period with blocks of beef cut along the grain were 55°C.—495 mg.; 75°C.—465 mg. With blocks of beef cut across the grain, 55°C.—360 mg.; 75°C.—350 mg.

Proteolysis Obtained With Papain at Room Temperature and the Usual Cooking Temperatures (Table 1): Twenty grams of beef were immersed for five minutes in 20 c.c. of papain solution containing 20 mg. of papain per cubic centimeter, and trichloracetic acid was then added to the samples that were not cooked. The samples heated in a 155°C.(311°F.) oven were placed on watch crystals after removal from the papain solution. After heating 25 minutes the meat was cooled to room temperature and trichloracetic acid added. The samples boiled in H_2O were heated in 40 c.c. of boiling H_2O for 10 minutes after being removed from the enzyme solution.

About three c.c. of papain solution adhered to the 20-gram blocks of beef after removal from the enzyme solution. The remaining 17 c.c. of papain solution and the water in which the beef was boiled were added to the beef after trichloracetic acid and used in diluting to the final volume.

Survival of Papain Cooked With Unground Meat (Table 2): Twenty-gram blocks of beef were heated with three c.c. papain paste containing 20 mg. of paste per cubic centimeter. The meat was heated with papain in the same manner as described for Table 1 and Fig. 1. After heating,

the mixtures were cooled, diluted to 100 c.c. with ice water, and disintegrated in the beater. One-tenth of this volume was then activated by the addition of five c.c. *2M* NaCN for four minutes at room temperature and then diluted to 100 c.c. with distilled H_2O. Total papain and not active papain was thus measured. One c.c. of this mixture was added to five c.c. of urea-hemoglobin solution, as used in the Anson method (1938). The digestion of hemoglobin was determined by the same color method used to determine proteolysis in meat.

Penetration of Papain Into Beef Muscle (Table 3): Round steak (4 to 5 cm. thick in Expt. 1; 2 to 2.5 cm. thick in Expt. 2) was placed in contact with a solution of papain containing 20 mg. of papain per cubic centimeter, so that the bottom surface of the steak was submerged to a depth of 10 mm. for 20 minutes at 23°C.(73.4°F.). The meat was then removed, wiped with blotting paper, and immediately frozen between blocks of solid CO_2. The lateral surfaces of the frozen steak were then trimmed off, and horizontal sections were cut from the remaining piece. The first slice off the bottom thus represented the surface of the steak previously in contact with the enzyme. Consecutive horizontal slices from the steak represented increasing distances from this surface of contact. In order to determine whether enzyme was present in the individual slices, each of these was weighed, disintegrated in a beater with four times the weight of neutral .02M NaCN (to activate any papain present), and then allowed to stand at room temperature overnight. Protein-free filtrates were then prepared and analyzed as before. The color value which was finally measured was the result of the digestion by papain overnight at room temperature of the liquefied beef muscle.

SUMMARY

Since tenderization is hard to determine quantitatively we have followed the digestion of meat by papain by means of a simple and accurate chemical method. This method is carried out in a few minutes without elaborate equipment, and several estimations can be made at one time.

Meat is digested by papain extensively and rapidly at 55 and 75°C. (131 and 167°F.) but very slowly at room temperature. At 55°C. papain is stable for 28 hours in the presence of meat. At 100°C.(212°F.) papain is rapidly destroyed in the presence of meat so that there is very little digestion above 100°C. Papain does not penetrate solid pieces of meat at room temperature.

When beef is ground the rate of digestion by papain is much increased.

The rate of proteolysis is not increased by previous cooking of either ground or unground meat.

In the usually recommended procedure for tenderizing meat, unground meat is painted with papain and then cooked. The experiments performed show that under such conditions papain would not penetrate very far into the meat, and the papain on the surface would be inactivated by heat before much digestion could take place.

We wish to express our gratitude to Dr. A. K. Balls for his numerous suggestions and his help in preparing this manuscript.

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ASCORBIC ACID CONTENT OF SOME CANNED GRAPEFRUIT JUICES PREPARED UNDER VARIOUS PROCESSING CONDITIONS[1]

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Grapefruit is produced in large quantities in the Rio Grande River valley of Texas, in Florida, and in California. Much of the fruit is packed and shipped to all parts of the country and served raw. Large quantities, however, are processed and the juice is pasteurized, canned, and put on the market for use throughout the entire year. Technical aspects of canning fruit juices, including grapefruit juice, have been discussed by Lueck and Pilcher (1941). Fresh grapefruit and canned grapefruit juice together constitute a tremendously important source of vitamin C in the diet. According to Bessey and King (1933) grapefruit contains 53 mg. of vitamin C per 100 grams of sample. Guerrant, Rasmussen, and Dutcher (1935) report that one sample of fresh grapefruit juice contained 27 mg. per 100 ml. and one sample of canned grapefruit juice contained 41 mg. of vitamin C per 100 ml. Holmes, Pigott, and Tripp (1941) found that fresh grapefruit juice averaged 48 mg. per 100 gm. and canned averaged 31 mg. Seventeen samples of six varieties of fresh grapefruit were found by Floyd and Fraps (1939) to contain from 23.6 to 49.1 mg. of vitamin C per 100 grams.

EXPERIMENTAL PROCEDURE

This study was undertaken to determine the influence of time elapsing between extraction and pasteurization of the juice, percentage of packing-house culls processed with the fresh fruit, type of extraction process employed, and exposure of the fruit on the tree to a hard freeze prior to harvesting, on the changes produced in the vitamin C content of canned grapefruit juice. With the generous co-operation of the leading citrus fruit canners of the Rio Grande Valley, samples were secured representative of various times of processing, methods of juice extraction, cull fractions, and pre-harvest freezes. Similar samples of grapefruit were extracted, heated, and canned similarly, except for variation in the time elapsing between cutting of the grapefruit and heating to pasteurization temperature, 87.8°C.(190°F.). Other similar samples of fruit were processed by different types of extractors—pressure screw, mechanical rotary grater, hand reaming burr, or automatic corrugated rollers—other condi-

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tions being kept as nearly the same as possible. The methods of extraction have been described by Lueck and Pilcher (1941). Representative samples of fruit containing approximately 90 per cent of packing-house culls (having skin blemishes or being too large or too small for shipping) were processed at the same time as samples of freshly harvested, tree-ripened fruit containing 10 per cent or less of packing-house culls. Samples of fresh fruit were packed late in January, 1940, just preceding a hard freeze which occurred in the orchards early in February. Representative samples of the same fruit which had come through the freeze were then packed in mid-February for comparison.

Samples for analysis were withdrawn with a small pipette from a beaker of freshly squeezed raw grapefruit juice or from a freshly opened can which had been vigorously shaken before opening. The juice was weighed in a weighing bottle and transferred to a 100-ml. volumetric flask, 35 ml. of two per cent HPO_3 and five per cent H_2SO_4 solution being used to wash out the juice quantitatively. The solution was then made up to volume with eight per cent acetic acid. After shaking the solution, five-ml. aliquots were titrated with a standardized solution of the sodium salt of 2, 6-dichlorophenolindophenol with the use of a microburette. A blank containing only the acids was run. The dye was standardized against fresh ascorbic acid, the ascorbic acid against iodine, and the iodine against arsenious oxide from the United States Bureau of Standards.

The first of the HPO_3-H_2SO_4 extracts of grapefruit juice, fresh or canned, were treated with hydrogen sulfide, allowed to stand 20 minutes, and swept out with carbon dioxide, before making up to volume with eight per cent acetic acid and titrating an aliquot. Since the hydrogen sulfide treatment did not cause any increase in the titrable ascorbic acid found in any case, it was evident that grapefruit juice does not contain any reversibly oxidized ascorbic acid. The hydrogen sulfide treatment was omitted, therefore, after the first four or five determinations.

DISCUSSION OF RESULTS

Twenty-one samples of fresh, raw juice from first-grade, tree-ripened grapefruit were analyzed for ascorbic acid. These juices contained an average of 41.2 mg. of ascorbic acid, with a maximum of 46.4 and a minimum of 38.3 mg. per 100 grams. The 109 samples of canned grapefruit juice averaged 33.7 mg. of ascorbic acid, with a maximum of 39 and a minimum of 26.3 mg. per 100 gm. The canned juices contained on the average 18.2 per cent less ascorbic acid than the fresh juices.

Inquiry concerning the practices followed in large-scale processing of grapefruit juice revealed considerable differences in the length of time the juice is held in storage after expression from the rind, pulp, and seeds before pasteurization. Some operators claim that ordinarily it is not more than one or two minutes from the time that the fruit is first cut until the juice is ready to seal in the cans. Other operators make a practice of allowing the extracted juice to stand for 30 minutes or even longer before placing it in the pasteurizer. Data on the ascorbic acid contained in canned juices which had stood for various lengths of time between extraction and pasteurization (Table 1) show that the freshly extracted juice

loses its ascorbic acid quite rapidly while standing in the tanks or passing through the line to the pasteurizing plant, and the standard deviation shows that these losses are significant. Losses of ascorbic acid can be decreased from 34.7 per cent to one-fifth of that amount by pasteurizing the juice immediately after cutting the fruit and extracting the juice.

It is well known that many canners of grapefruit juice rely for the great bulk of their fruit supply on packing-house culls. Culls consist of fruit which, because of skin blemishes, irregularities of size, or excessively thick skin, has been culled out as unsuitable for shipment as fresh fruit

TABLE 1

Ascorbic Acid in Canned Grapefruit Juices Extracted From Fresh Fruit and Kept Standing for Various Lengths of Time Before Pasteurizing

Time between extraction and pasteurization	Ascorbic acid	Average ascorbic and standard deviation	Average loss of ascorbic acid during canning compared with fresh juice containing an average of 41.2 mg. per 100 gm.
min.	*mg./100 gm.*	*mg./100 gm.*	*pct.*
3.5 or less	39.0, 39.0, 38.8	38.5	
	38.4, 38.4, 39.3		6.6
	38.0, 37.6, 38.2	S.D. 0.54	
	38.0, ..., ...		
5	36.7, 35.8, 36.1	36.6	
	37.0, 37.0, 36.9		11.2
	36.9, ..., ...	S.D. 0.48	
20	28.0, 30.7, 30.7	29.0	
	28.2, 29.1, 28.8		29.6
	28.1, 29.3, 28.5	S.D. 1.04	
30	27.0, 26.7, 27.0	26.9	
	26.6, 27.4, 27.5		34.7
	26.3, 26.6, ...	S.D. 0.42	

in a fresh-fruit packing house. As a rule, culled fruit has been harvested two or three days prior to its reception at the juice canning plant. It is mixed at the canning plant with small percentages of fruit coming fresh from the groves. Other canners, however, get 90 per cent or more of the fruit they use directly from the trees and process it immediately. Juice from such fruit is extracted, cooked, and sealed in the can within less than 12 hours from the time of picking. This fruit is subjected to a minimum of handling and minor bruising owing to handling.

Fourteen samples of canned juices prepared from fresh fruits, containing 10 per cent or less of culls, contained on the average 36.1 mg. of ascorbic acid per 100 gm. of juice. Twelve samples of canned juices prepared from culls, i. e., consisting of approximately 90 per cent culls, contained an average of 29.5 mg. of ascorbic acid per 100 gm. of juice. Vitamin C losses during the canning of juice from fruit directly from the trees were slightly less than one-half the losses during the handling and processing of fruit that passed through fresh-fruit packing plants (Table 2).

Early in February, 1940, a cold wave spread over the Rio Grande Valley and sub-freezing temperatures prevailed for two or three days. Several samples of canned grapefruit juice, some of which had been prepared from fruit gathered before and some after the freeze, were received

TABLE 2

Ascorbic Acid in Canned Grapefruit Juices Prepared From Fruits Containing Different Percentages of Packing-House Culls in Freshly Harvested Grapefruit

Packing-house culls in fresh grapefruit	Ascorbic acid	Average ascorbic acid	Average difference in ascorbic acid of canned and fresh juice containing an average of 41.2 mg. per 100 gm.
pct.	*mg./100 gm.*	*mg./100 gm.*	*pct.*
10 or less	36.5, 37.0 36.5, 36.1 37.0, 36.3 36.6, 36.6 35.7, 36.0 35.4, 35.7 35.0, 35.0	36.1 S.D. 0.66	12.4
90 (approx.)	28.0, 29.8 28.5, 28.2 28.0, 30.0 27.8, 31.0 31.4, 29.0 30.5, 31.2	29.5 S.D. 1.36	28.4

at Sam Houston Laboratory for vitamin C analysis. Six samples of canned juices from fruit picked before the freeze averaged 36 mg. of ascorbic acid per 100 gm. of sample, while six canned juices from fruit gathered after the freezing weather averaged 31.1 mg. per 100 gm. Losses of ascorbic acid during the processing of fruit which had gone through the freeze

TABLE 3

Ascorbic Acid in Canned Grapefruit Juices Prepared From Fruits Gathered Before and After Sub-Freezing Weather of Early February, 1940

Time of picking fruit	Ascorbic acid	Average ascorbic acid	Average difference in ascorbic acid of canned juice compared with fresh juice containing an average of 41.2 mg. per 100 gm.
	mg./100 gm.	*mg./100 gm.*	*pct.*
Before freeze	37.0, 36.7, 36.0 36.0, 35.1, 35.4	36.0 S.D. 0.73	12.6
After freeze	32.0, 30.3, 30.5 30.5, 31.6, 31.4	31.1 S.D. 0.71	24.8

were almost double the losses of fruit gathered before the freeze (Table 3), and statistical analysis shows that the difference is highly significant.

Some canners of the Rio Grande Valley area generally employ one or a combination of four different methods of extracting grapefruit juice from the fruit.

In one method, involving screw extraction, the fruit is quartered and the quarters are held in place on the outer extremity of a revolving wheel. These quarters are then passed over a roller which operates in a direction opposite to that of the wheel. In this way all pulp, seeds, and juice are removed from the rind. The rind is then taken on a belt to the disposal plant. The juice is squeezed out from the rag and seeds in a screw extractor.

In a second method the fruit is halved and the halves are conveyed on a belt to a reaming table on which there are several burr-heads similar to

TABLE 4

Ascorbic Acid in Canned Grapefruit Juices Separated From Fruit Solids by Various Types of Extractors

Type of extractor	Ascorbic acid	Average ascorbic acid	Average loss of ascorbic acid during canning fresh juice containing an average of 41.2 mg. per 100 gm.
	mg./100 gm.	*mg./100 gm.*	*pct.*
Screw (pressing)	30.0, 35.6, 35.1 35.9, 35.2, 29.5 29.6, 33.6, 34.0 33.5, ..., ...	33.2 S.D. 2.55	19.4
Burr (hand reaming)	33.5, 32.5, 32.5 31.6, 34.7, 36.4 34.2, 30.4, 35.7 35.7, ..., ...	33.7 S.D. 1.96	18.2
Corrugated rollers (automatic)	36.5, 35.9, 35.9 35.8, 35.4, 35.9 37.3, 35.7, 32.5 36.1, ..., ...	35.7 S.D. 1.24	13.3
Rotary grater (mechanical)	37.0, 37.2, 34.5 35.7, 37.4, 36.3 37.0, ..., ...	36.4 S.D. 1.04	11.7

those in fruit-juice extractors in drug stores or home kitchens. These burrs are rotated at high speed while the halves are held over and pressed down on the burr-head by hand until the burr-head touches the rind. Then the rind is discarded and the juice is conveyed to and run through a series of two or more moving or shaking screens.

A third method of operation consists of quartering the fruit and rolling the quarters between two corrugated rollers. The rollers are adjusted sufficiently close together to express the juice and meat from the rind, but not sufficiently close to produce any appreciable pressure on the rind. The hulls are kicked out of the machine. The juice runs straight down to troughs which convey it to a series of revolving screens. These screens separate the juice from the seeds and pulp. This system is referred to as an automatic extractor.

A fourth type is a mechanical juice extractor. Fruit is fed to this extractor individually. Each grapefruit is halved and each half is partially cut again so that the pieces fit into a pocket of a large wheel. This wheel

then brings the fruit into contact with rotary graters. The seed, rag, and juice are grated out and conveyed to a series of portable screens which separate the juice from the seeds, rag, and finer particles.

The ascorbic acid contents of several canned grapefruit juices which had been processed in the respective types of juice extractors are shown (Table 4). Ten canned juices which had been prepared in a screw extractor averaged 33.2 mg. of ascorbic acid per 100 gm. Ten cans of juice which had been hand reamed on a burr-head averaged 33.7 mg. per 100 gm. Ten canned samples which had been run through automatic corrugated rollers averaged 35.7 mg. per 100 gm. Seven cans of juice extracted in a mechanical rotary grater averaged 36.4 mg. per 100 gm. Statistical analysis by Fisher (1928) shows that, while the differences are small, vitamin C is significantly lower in the screw or pressing type of apparatus than in corrugated roller or rotary grater types. No other differences are significant.

SUMMARY

Canned grapefruit juice averaged 33.7 mg. ascorbic acid per 100 gm. in 109 samples compared with 41.2 mg. in 21 samples of fresh raw juice from first-grade, tree-ripened fruit. Freshly extracted juice did lose 11.2 per cent of its ascorbic acid if allowed to stand five minutes before pasteurizing and 34.7 per cent in 30 minutes. Juice from culls contains less ascorbic acid than first-grade fruit. Freezing also decreases the percentage in the juice. Juice prepared with machines using corrugated rollers or a rotary grater contained a little more ascorbic acid than that prepared by screen pressing or hand reaming.

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EFFECT OF FREEZING RATE ON QUALITY OF BROILED STEAKS

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In recent years considerable discussion has appeared in the trade and technical journals as well as in extension and experiment station circulars and bulletins as to the numerous benefits of quick-freezing. Cook, Love, Vickery, and Young (1926) concluded that the rate of freezing was the most important factor affecting rate of drip. Empey (1933) claimed, however, that the rate of freezing had no influence on susceptibility to drip but that drip was largely a function of the hydrogen-ion concentration. Moran and Hale (1932) found that the length of storage and thawing temperatures, 10 and 1°C.(50 and 33.8°F.), had no appreciable effect on drip, while the storage temperature and fluctuation in temperature produced a marked effect. Paul and Child (1937) stated that frozen beef thawed at 24 to 25°C.(75.2 to 77°F.) at 65 per cent relative humidity gained weight rather than losing it as does beef thawed at 175°C. (347°F.). They also found that the total moisture, drip, and tenderness of cooked beef were unaffected by freezing or by different thawing temperatures. In this case the authors report that the freezing temperature was held at —18.2°C.(—.6°F.) and that it took 25 hours for the interior of the meat to reach this temperature. Hankins and Hiner (1940) report that low-temperature freezing, —40 and —23.3°C.(—40 and —10°F.), had greater tenderizing effect than freezing at —6.7°C.(20°F.). Cook and White (1941) report that a temperature of —18°C.(—.4°F.) or lower is necessary for the protection of pork for storage periods of approximately one year. Ramsbottom and Koonz (1939) found that irrespective of freezing temperatures, —12.2, —23.3, —34.4, and —58°C.(10, —10, —30, and —50°F.), with large rib cuts the difference in drip was insignificant. Steaks frozen at —6.7, —23.3, and —58°C.(20, —10, and —50°F.) showed a decreasing amount of drip with the lower freezing temperatures. The authors state that this difference in drip may be explained on the basis of extra-fiber and intra-fiber freezing.

At Iowa (1941) where beef roast and steaks were frozen at —17.8 and —23.3°C.(0 and —10°F.) it was found that there were no significant differences in total shrinkage, acidity values, and palatability of lean between beef roasts frozen at the two temperatures. The shrinkage of steaks frozen at —10°F. was, however, markedly greater than those frozen at 0°F. The losses from steaks were greater and less uniform than in the case of the roast. Approximately one-third of the total loss from steaks stored one year occurred the first month. Cooking losses were inversely proportional to the storage losses for the various periods of time. The roasts frozen at —10°F. were more tender than those frozen at 0°F., while there was no

difference between the steaks in this respect. The steaks also scored lower in juiciness than the roasts.

EXPERIMENTAL PROCEDURE

During the past two years the Idaho Agricultural Experiment Station has studied the effect of quick-freezing and slow-freezing steaks from beef rounds and from the legs of pork and lamb. The meat studied consisted of steaks from two pairs of U. S. Commercial beef rounds weighing an average of 90 pounds per pair, rump off; two pairs of U. S. Good lamb legs weighing 16 pounds per pair; and two pairs of U. S. Choice pork legs weighing 24 pounds per pair. The beef, lamb, and pork carcasses

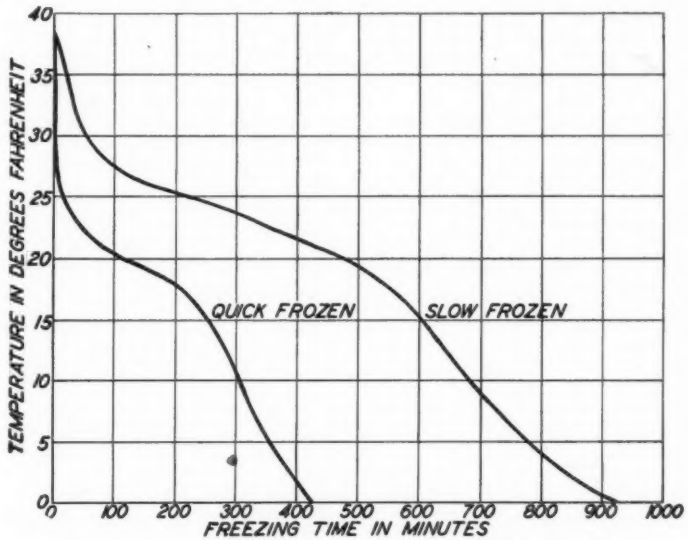

FIG. 1. Rate of freezing for slow-frozen and quick-frozen steaks.

were aged ten, five, and two days, respectively, after slaughter. In case of the beef rounds, they were boned and separated into top and bottom cuts. Steaks .6 inch in thickness were cut and wrapped in 30-pound test, double waxed, freezer wrapper paper and then double wrapped with regular butcher paper. The steaks weighed an average of 350 to 400 grams before wrapping. Each cut from the left and right side was paired as to location; those on the left side were quick-frozen and those on the right side were slow-frozen.

Freezing: To facilitate freezing a steel chamber four by four by five feet in size and having four screen trays was used. The chamber was completely enclosed except for a screen bottom which was set one foot off the floor, and the top which was fitted with a circulating fan. The steaks were then spread over the screens and at four different locations thermocouples were inserted into the center of the steaks. For the slow-frozen steaks the chamber was put into a room held at —17.8°C.(0°F.). With the quick-

frozen steaks the room was held at —26.1°C.(—15°F.). Frequent temperature readings were taken from the thermocouple leads at regular intervals until the average internal temperature of the steaks reached —17.8°C.

It is to be noted that an average of approximately seven hours and 10 minutes was required for the interior of the quick-frozen steaks to reach a temperature of —17.8°C. This would be a cooling rate of 2.9°C. (5.3°F.) per hour. In case of the slow-frozen steaks it took 15 hours and 20 minutes for the average internal temperature of the steaks to reach —17.8°C. This would be an average cooling rate of 1.4°C.(2.5°F.) per hour or approximately one-half the quick-freezing rate.

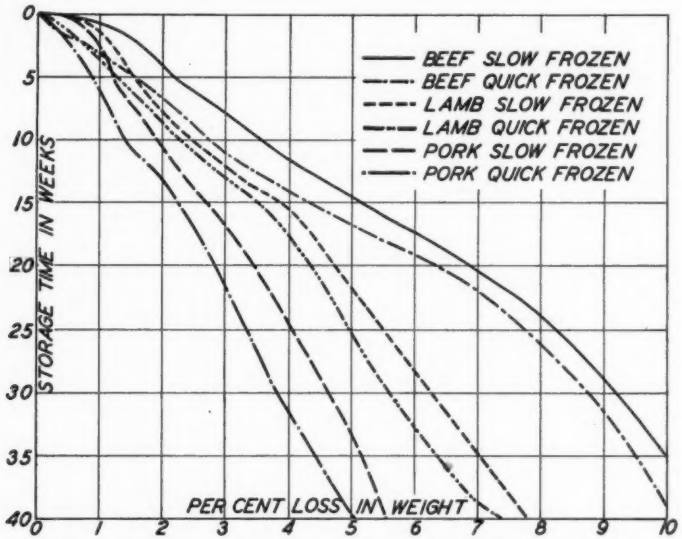

FIG. 2. Evaporation rate of slow-frozen and quick-frozen steaks.

Storage: After freezing, the meat was removed and placed in locker storage for a period of 39 weeks. During the storage period a constant check was kept on the relative humidity and temperature. The relative humidity ranged from 85 to 90 with an average of 87 per cent. Generally, the variation was not to exceed one per cent in any one 24-hour period. The temperature was held nearly constant at —14.4°C.(6°F.) during the storage period. The maximum daily variation was not to exceed 1.7°C. (2°F.), recorded on the thermograph. Weights were taken at 14 different periods to check on the evaporation rate of the stored meat.

Cooking Procedure: At the end of the storage period the steaks were removed to be broiled according to standard methods. With each pair of steaks one was allowed to thaw at room temperature while the other was kept frozen until broiling time. The steaks were broiled to an internal temperature of 70°C.(158°F.) in an oven preheated to 177°C.(350.6°F.). After broiling, sections of the adductor muscle were served to the palata-

bility committee. Data were collected on the drip and evaporation losses during broiling.

EXPERIMENTAL RESULTS

The evaporation during 39 weeks of storage was 4.9, 7.0, and 9.8 per cent, respectively, for the quick-frozen pork, lamb, and beef; and 5.5, 7.6, and 10.3 per cent, respectively, for the slow-frozen pork, lamb, and beef (Fig. 2).

It is to be noted that the evaporation rate on the quick-frozen steaks is consistently lower than in the case of the slow-frozen steaks. The highest evaporation rate was found in the relatively lean beef steaks while the smallest evaporation rate was found with the rather fat pork steaks. The lamb steaks, which were intermediate in the degree of fatness, showed an intermediate evaporation rate.

Cooking Losses: To determine if there was any difference in the cooking losses of the steaks allowed to thaw and steaks broiled while frozen, one-half were broiled each way.

The smallest total loss (drip and evaporation) occurred in the quick-frozen steaks, broiled while still frozen, and the largest loss occurred in the slow-frozen steaks, thawed before broiling (Table 1). Figures for the steaks which were quick-frozen and cooked thawed as well as slow-frozen and cooked frozen would indicate that the quick-freezing was of greater importance in preventing loss than the cooking while frozen.

Somewhat higher losses for drip than for evaporation are indicated (Table 2). Broiling the beef steaks while frozen also appears to be advantageous.

It will be noted that the lamb was the most palatable and the beef was the least palatable while the pork was intermediate (Table 3). There were no significant differences in palatability between the slow-frozen and quick-frozen steaks, nor between the steaks broiled thawed or frozen.

DISCUSSION

Practically all of the work reported in the literature has been obtained under such widely varying conditions that it is rather difficult to compare the results. One of the greatest difficulties encountered in discussing the rate of freezing is that while the room temperature at which the freezing takes place may be reported, no mention is made of the actual freezing rate. It is well known that two cuts of meat may be frozen in markedly different lengths of time even under the same temperature conditions owing to variations in the mechanical setup affecting the rate of heat transfer.

In the work reported in this paper steaks .6 inch in thickness showed an evaporation rate ranging from approximately five to 10 per cent over a period of 39 weeks. The effect of the size and shape of the cut on dehydration is clearly shown since prime rib roasts weighing an average of seven pounds and stored under identical conditions for the same length of time showed a total evaporation loss of one and one-half per cent. It is to be noted that while the evaporation is not greatly higher for the slow-frozen steaks as compared with the quick-frozen, it is nevertheless consistent for beef, pork, and lamb.

TABLE 1

Percentage Total Weight Loss in Steaks Cooked Thawed and Frozen

Treatment	Beef		Pork		Lamb	
	Quick-frozen	Slow-frozen	Quick-frozen	Slow-frozen	Quick-frozen	Slow-frozen
Cooked thawed	30.98	42.31	26.80	29.87	33.26	34.97
Cooked frozen	27.09	35.14	19.65	28.83	32.41	34.20

TABLE 2

Percentage of Drip and Evaporation From Steaks Broiled Thawed and Frozen

Treatment	Beef		Pork		Lamb	
	Drip	Evaporation	Drip	Evaporation	Drip	Evaporation
Broiled thawed	19.81	17.30	20.45	7.95	20.77	13.70
Broiled frozen	17.99	13.36	16.06	8.48	20.91	14.82

TABLE 3

Average Palatability Scores for Broiled Beef, Pork, and Lamb Steaks[1]

Scoring points	Beef		Pork		Lamb		
	Broiled thawed	Broiled frozen	Broiled thawed	Broiled frozen	Broiled thawed	Broiled frozen	
Aroma							
Intensity	4.8	5.0	5.0	5.0	6.0	6.0	Quick-frozen
	5.2	5.0	5.0	5.0	5.0	5.5	Slow-frozen
Desirability	5.6	5.2	5.3	5.0	6.0	5.5	Quick-frozen
	5.5	5.6	6.0	5.0	6.0	5.5	Slow-frozen
Texture	5.6	5.2	5.0	5.0	6.0	5.0	Quick-frozen
	5.5	5.6	4.7	4.7	6.0	5.0	Slow-frozen
Flavor of lean							
Intensity	4.9	4.4	5.0	5.0	5.5	5.5	Quick-frozen
	4.6	4.6	5.0	4.7	5.5	5.5	Slow-frozen
Desirability	3.8	3.8	4.7	5.0	5.5	5.5	Quick-frozen
	4.0	3.6	4.7	4.0	5.0	5.5	Slow-frozen
Tenderness							
Intensity	4.8	5.0	4.7	5.0	6.0	5.5	Quick-frozen
	4.8	4.4	4.7	4.3	5.5	6.5	Slow-frozen
Quality of juice							
Intensity	3.8	3.9	4.3	4.0	4.5	4.5	Quick-frozen
	4.2	3.7	4.0	3.3	4.5	5.0	Slow-frozen
Desirability	3.9	3.9	5.0	4.7	4.5	5.5	Quick-frozen
	4.4	3.8	4.7	4.0	4.5	5.0	Slow-frozen
Quantity of juice							
Intensity	3.0	2.4	3.3	2.7	4.5	4.0	Quick-frozen
	2.7	2.8	2.3	3.0	4.0	5.0	Slow-frozen
Desirability	5.0	3.9	5.3	5.7	5.0	5.0	Quick-frozen
	4.6	4.5	5.3	4.0	4.0	3.5	Slow-frozen

[1] Palatability scores are the averages for five judges and range from 0 to 7 with increasing desirability.

It is also to be noted that the cooking losses (evaporation and drip) ran considerably higher on the slow-frozen steaks than on the quick-frozen steaks. This amounted to a 37-per cent greater loss on the beef steaks cooked after thawing and a 30-per cent greater loss on the beef steaks cooked while frozen. In the case of the lamb steaks there was approximately a five-per cent greater cooking loss with slow-frozen steaks as compared with the quick-frozen steaks for both methods of cooking. The cooking losses for pork also showed an advantage of the quick-frozen over the slow-frozen steaks although there is a very large difference with the two methods of cookery, there being 11 per cent greater loss with the pork cooked thawed and 47 per cent greater loss with the pork cooked frozen.

As regards the palatability scores, no significant difference was to be found between the quick-frozen and slow-frozen steaks nor between the steaks cooked while frozen or thawed.

SUMMARY

Studies on the relative advantage of quick-freezing and slow-freezing of pork, lamb, and beef reveal that the slow-frozen meat had a higher evaporation rate during storage than the quick-frozen. The smallest cooking loss (drip and evaporation) occurred in the quick-frozen steaks broiled while frozen and the largest loss occurred in the slow-frozen steaks, thawed before broiling. It appears desirable that thin cuts of meat should be quick-frozen and cooked while still frozen to prevent a high loss in evaporation and drip even though there is no apparent effect on the palatability of steaks prepared this way.

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EFFECT OF ORANGE AND LEMON JUICES ON ACTIVITY OF PROTEOLYTIC ENZYMES

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It was observed in some *in vitro* experiments (unreported) that the addition of orange juice to solutions containing bromelin or papain definitely accelerated the activity of these enzymes. Since the orange juice in the absence of added bromelin or papain exhibited only slight proteolytic activity, and since the activating factor was heat labile, we believed the most logical explanation to be that fresh orange juice contains a coenzyme capable of activating both bromelin and papain.

These results, particularly in view of the clinical results obtained by Sansum and Gray (1929), caused us to question whether peptic activity might be accelerated by orange or lemon juice. Further work thus prompted has resulted in the accumulation of considerable data here presented in brief form. Certain conclusions are stated, but in any attempt to evaluate these data or the conclusions reached it should be borne in mind that these are *in vitro* experiments only.

Either sausage casings or cooked egg albumen was used as the substrate. The ribbon variety of surgical suture was tried as substrate, but because of its extremely dehydrated and otherwise denatured condition it was found to be unsatisfactory. In several experiments parallel determinations were made in which citric acid solutions containing the same weight of anhydrous citric acid as the lemon juice were compared with lemon juice in their effect on pepsin. In making these determinations the lemon juice and the corresponding citric acid solutions were brought to the same pH [1] by adjustment with HCl.

EXPERIMENTAL PROCEDURE

Narrow, uniform strips (a) of sausage casings [2] and (b) of cooked egg albumen were used as substrate materials. These strips were weighted by attaching small paper clips to the bottom or free end of the strip. The strips were suspended in test solutions containing the enzyme together with lemon juice and/or other ingredients. Pepsin [3] was used at concentrations of .067 and .33 per cent. Each test solution was finally made up to a total volume of 30 ml. For convenience, test mixes containing several 30-ml. portions were sometimes prepared, allowing for a division into as many 30-ml. aliquots as needed for duplicate tests.

The lemon juice used was hand burred on a glass burrer, and strained through a 20-mesh sieve. In some instances the juice was deaerated before

[1] All pH values were determined by use of the glass electrode.

[2] Of sheep-gut origin, commercially available, preserved in salt. The casings were desalted, washed with alcohol and ether, and dried before cutting into strips.

[3] Pepsin 1:10,000, granular, Reference No. 306560, Difco Laboratories, Detroit, Michigan.

use; but deaeration did not result in any observed effect on peptic activity.

When the term "six per cent citric acid solution" is used in either the tables or the text it refers to a solution containing six grams of anhydrous citric acid per 100 ml.

In making comparisons between test mixes containing lemon juice and those containing citric acid, sufficient citric acid solution was used in the latter to give the same weight of citric acid as contained in an equal volume of the lemon juice solution. The citric acid content of the lemon juice was determined by analysis.

Control test mixes were prepared from which lemon juice was omitted but which were brought to the same pH as the other mixes of the series by adjustment with HCl. The activity of the enzyme and any acceleration of this activity owing to the presence of lemon juice was estimated by observing the time at 40°C.(104°F.) required for the strips being compared to break.

The change in activity owing to the addition of lemon juice or other ingredient under test has been calculated to a per cent decrease (acceleration) or increase (retardation) in the time required for the strip placed in the test solution containing these added ingredients to break as compared with a similar strip contained in the control test solution. For purposes of these experiments the breaking of the test strip has been taken arbitrarily as the end point; although it is recognized that such breaking does not necessarily indicate the completion of peptic action.

The effect of pH upon the tests was studied by making a series of experiments in which the pH of the different experiments of the series was varied.

Sausage-casing strips were used for obtaining a considerable portion of the data reported; but because of the very great physical differences found in sausage casings their use is not recommended for future work. Since properly prepared strips of egg albumen are not subject to excessive fluctuations, they are much more suitable. However, to insure close agreement between duplicates it is necessary that very close attention be given to details of technique in preparing the egg-albumen strips. Therefore, procedure for their preparation and use is given in considerable detail.

Preparation and Use of Cooked Egg Albumen Strips: Add one part distilled water to three parts fresh egg white and beat gently with a spoon for three minutes. Deaerate under suction until foam subsides and filter through two thicknesses of new cheesecloth. Store in the cold but do not freeze.

From a piece of red (heat resisting) sheet gasket rubber about 1.5 mm. in thickness cut a rectangular strip 75 x 25 mm., slightly smaller than an ordinary microscope slide. Using a sharp knife cut out the center portion (65 x 17 mm.) of the strip. Rub the resulting gasket with paraffin wax to make its surfaces smooth and water repellent. Cut strips of ordinary Cellophane 170 x 26 mm. and place in a water bath at 80 to 85°C. (176 to 185°F.) for 15 minutes, remove, wash off any film of softened material which may be present and use the moist strips for wrapping two microscope strips, using one strip for each slide. The ends of the strips will overlap about 10 mm. and in the moist condition will adhere sufficiently. The prepared rubber gasket is placed on the smooth side of one of the Cellophane wrapped slides. The second wrapped slide is superimposed over the first in such a manner as to cover the gasket except that one end is left open to a distance of about five mm. In this position both slides are carefully fitted to the gasket by gentle pressure. Elevate and support the open end about 10 mm. above the table top.

Add the prepared egg white slowly from a pipette, filling the space enclosed by the gasket and the two slides. If entrapped air bubbles should persist, remove them by tipping the slides or with the aid of a fine wire. Continue adding egg white until the open end of the gasket is filled to overflowing, then slip the top slide forward until it is superimposed above the other throughout its length. Fasten the slides firmly in this position by wrapping several times with a light string and tying securely.

Cook the contained egg white by placing the tied package in a water bath at 85 to 87°C.(185 to 188.6°F.) and holding for 30 minutes. Remove, and place in a cold water bath, 20 to 25°C.(68 to 77°F.), for two minutes. Take the string wrapping from the package, open back the overlapped ends of the Cellophane wraps and place the slides with either face down on a smooth surface. Insert a knife blade between the top slide and its Cellophane wrap and carefully pry the slide loose. Start at one end of the top Cellophane strip and gently raise it, thus removing it from contact with the egg-albumen ribbon. Remove the gasket and ribbon from the bottom Cellophane strip by starting at one end and gently pulling the gasket upward, holding the Cellophane strip in place if necessary. Place the gasket and its contained ribbon on a clean, moistened slide; remove the gasket from the adhering ribbon by running a sharp, thin blade between them. The ribbon of cooked egg albumen is ready to be cut into strips for immediate use or it may be placed in a moist chamber and stored in the icebox for later use.

To cut the strips apply a sharp, long blade; avoid drawing action which would cause distortion or tearing. Remove and discard a narrow strip (about 1.5 mm. wide) from each edge of the ribbon. Cut strips for use the length of the ribbon and with a width of about two mm. So cut the strips that their width will be as uniform as possible, one with the other, throughout their length.

Tubes: Cut off 1 x 8-inch tubes leaving them 110 mm. in length (over-all measurement).

Hooks: Using five mm. glass tubing (outside measurement) seal off one end of 130-mm. lengths. Bend into S shape with the over-all height of the "S" being about 40 mm.; the bottom curve of the "S" being the closed end forming a hook from which the strip is to be suspended. The over-all width of this hook should be 18 to 20 mm. which will allow easy entry into the test tube without excessive clearance. The open end of the "S" provides for hanging over the edge of the test tube.

When the test solutions have been placed in the tubes and their temperatures are being adjusted the strips should be placed on the glass hooks. Suspend the glass hooks from the rim of a one-liter beaker with the lower hook extending outside the beaker. Run a blade under one end of a strip and lift it sufficiently so that it may be taken up by the fingers. Hang the strip from its middle over the lower hook. Bring the ends of the strip together and tie about five mm. from the ends. Tie with a surgeon's knot using a 170-mm. length of woof from new cheesecloth. Draw the knot snug but not so tight as to cut the strip. Tie a square knot at a sufficient distance from the surgeon's knot to make a loop of the woof about seven mm. in length. Remove excess woof with sharp scissors, and attach a No. 3 Gem paper clip to the loop.

When all the strips to be used in the experiment have been made ready and the temperature of the test solutions has been adjusted, place the strips in the tubes, holding them in place by hanging the upper hook over the edge of the test tubes. Add the strips rapidly but with care, and record the time of addition. Maintain the temperature of the test solutions at 40°C.(104°F.) in an open water bath or in a constant temperature incubator with a glass door. Keep the strips under constant observation until all have broken, and as each breaks record the time.

DATA

The data obtained by the use of lemon juice and by citric acid solutions containing the same amount of citric acid, using sausage-casing strips as substrate, are shown (Fig. 1). The parallel trials with lemon juice and citric acid were conducted in order to determine the extent to which the citric acid content of lemon juice was responsible for changes in peptic activity. Above pH 1.5 (1.5 to 2.4) both lemon juice and citric acid

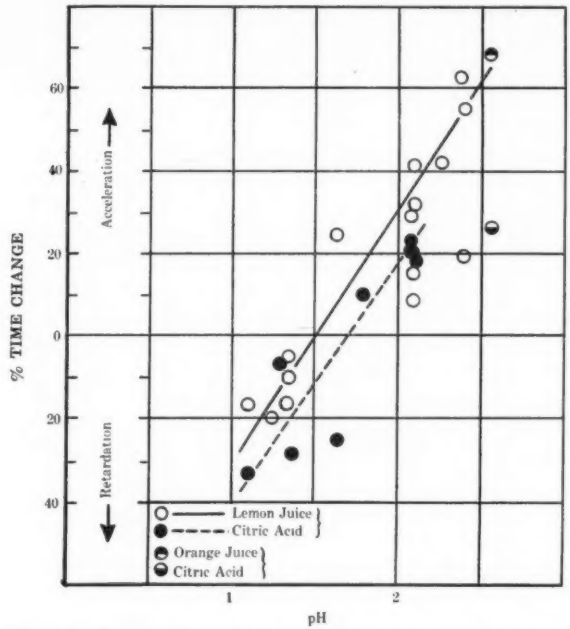

FIG. 1. Effect of lemon juice and of citric acid solutions on peptic activity as influenced by pH, using sausage-casing strips as substrate.

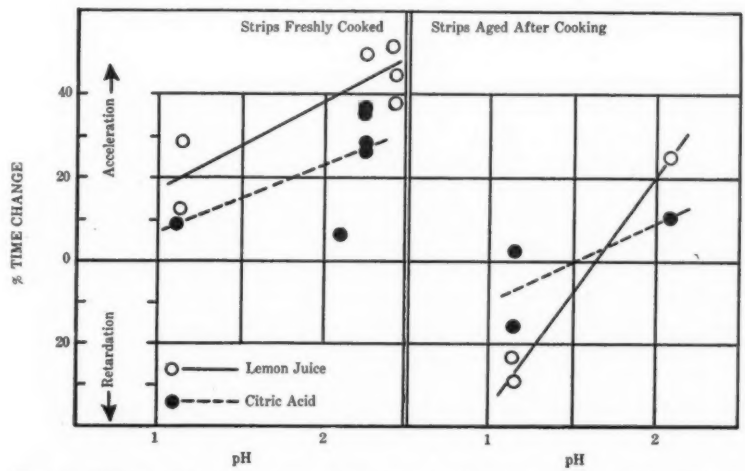

FIG. 2. Effect of lemon juice and of citric acid solutions on peptic activity as influenced by pH, using cooked egg-albumen strips as substrate.

increased the activity of pepsin; while below pH 1.5 (1.5 to 1.1) both retarded peptic activity.

A comparison of the two curves shows that lemon juice accelerates peptic activity somewhat more in the higher pH range and retards somewhat less in the lower pH range than does citric acid.

In the experiment in which orange juice was used at a pH of 2.56 both the orange juice and the corresponding amount of citric acid solution increased the activity of pepsin, but the increase in the presence of orange juice was considerably greater than in the presence of citric acid solution.

The effect of lemon juice and of citric acid solutions on peptic activity, and the influence of pH as determined by the use of cooked egg-albumen strips as substrate are shown (Fig. 2). When the strips were freshly cooked both lemon juice and citric acid increased the activity of pepsin throughout the pH range examined (1.1 to 2.4).

With aged, cooked strips as substrate at pH 1.1 both lemon juice and citric acid retarded, while at pH 2.1 both accelerated the action of pepsin. The strips were aged by placing in a moist chamber and storing at 10°C. (50°F.) for 72 hours.

(Tables 1 to 3 are representative of several experiments and show details. Tables 4 and 5 show details of experiments demonstrating the effect in the presence of citric acid: of ascorbic acid, ascorbic acid + iron and of iron as compared with citric acid alone and with lemon juice alone. Table 6 shows details of an experiment which demonstrates that lemon juice contains a proteolytic enzyme capable of the slow digestion of cooked egg albumen.)

DISCUSSION

The data presented demonstrate that in the presence of pepsin lemon juice accelerates the digestive process. What part of this is due to an activation of pepsin by the juice is not known but it is probable that the observed effect is largely accounted for by the citric acid contained in the juice. Acceleration of the digestive process in excess of that accounted for by the citric acid activation is probably due to the normal presence of some unidentified proteolytic enzyme in fresh lemon juice, since in the absence of any added pepsin or other proteolytic enzyme fresh lemon juice was observed to digest protein materials.

Egg albumen which has aged after cooking appears to change in character. In this denatured form peptic action in the presence of lemon juice follows about the same trend as shown by sausage-casing strips under similar test conditions. Also, it is probable that the sausage-casing material is somewhat denatured by the addition of salt and by partial drying. Possibly a common change is reflected in both instances by retardation of peptic activity at low pH.

Haggard and Greenberg (1941) concluded from *in vivo* studies in which various canned fruit juices (including grapefruit and orange) were used that these fruit juices exhibited no proteolytic activity. However, since their experiments were based on the use of canned juice, their results are not necessarily comparable to those reported here, as it is almost certain that the juices were heat sterilized in the canning process.

TABLE 1

Effect of Lemon Juice and Citric Acid on Peptic Activity, Using Sausage-Casing Strips as Substrate

Test	Lemon juice	6% citric acid solution	HCl .1 N	H_2O	Pepsin	pH	Time required for strip to break	Decrease in breaking time; acceleration
	ml.	*ml.*	*ml.*	*ml.*	*mg.*		*min.*	*pct.*
A	None	None	3.1	26.9	20	2.09	12.0	.....
B	10	None	4.4	15.6	20	2.07	8.5	29.2
C	None	10	0.2	19.8	20	2.07	9.5	20.8

TABLE 2

Effect of Lemon Juice on Peptic Activity, Using Freshly Cooked Egg-Albumen Strips as Substrate

Test	Lemon juice	HCl .02 N	H_2O	Pepsin	pH	Time required for strip to break	Decrease in breaking time; acceleration
	ml.	*ml.*	*ml.*	*mg.*		*min.*	*pct.*
A	10	5	15	100	2.45	66	.....
B	Duplicate of A					63	.....
C	Duplicate of A					61	.....
					Average	63.3	44.5
D	None	14	16	100	2.45	127	.....
E	Duplicate of D					102	.....
F	Duplicate of D					113	.....
					Average	114	Control

TABLE 3

Effect of Lemon Juice and Citric Acid on Peptic Activity, Using Freshly Cooked Egg-Albumen Strips as Substrate

Test	Lemon juice	6% citric acid solution	HCl .1 N	H_2O	Pepsin	pH	Time required for strip to break		Average decrease in breaking time; acceleration
	ml.	*ml.*	*ml.*	*ml.*	*mg.*		*min.*	*av.*	*pct.*
A	10	None	7	13	100	2.08	35	.....	.....
B	Duplicate of A		.....	.....	.....	.....	44	39.5	30.1
C	None	9.4	1.8	18.8	100	2.08	43	.....	.....
D	Duplicate of C		.....	.....	.....	.....	62	52.5	7.1
E	None	None	4.6	25.4	100	2.10	59	.....	.....
F	Duplicate of E		.....	.....	.....	.....	54	56.5	Control

TABLE 4

Effect of Lemon Juice as Compared With Citric Acid, Citric Acid + Ascorbic Acid, Citric Acid + Ascorbic Acid + Iron, and Citric Acid + Iron on Peptic Activity, Using Freshly Cooked Egg-Albumen Strips as Substrate

Test	Lemon juice	6% citric acid solution	HCl .1 N	H_2O	Ascorbic acid [1]	$FeSO_4 \cdot 7 H_2O$ [2]	Pepsin	pH	Time required for strip to break	Decrease in breaking time; acceleration
	ml.	*ml.*	*ml.*	*ml.*	*mg.*	*mg.*	*mg.*		*min.*	*pct.*
A	10	None	4.7	15.3	None	None	100	2.23	15	50.0
B	None	8.1	0.2	21.7	None	None	100	2.23	.... [3]	.....
C	None	8.1	0.1	21.8	21.9	None	100	2.24	19	36.7
D	None	8.1	0.1	21.8	21.9	34.5	100	2.23	23	23.3
E	None	8.1	0.1	21.8	None	34.5	100	2.23	23	23.3
F	None	None	3.7	26.3	None	None	100	2.23	30	Control

[1] Eastman Kodak Company, ascorbic acid for chemical purposes No. 4640. [2] Baker's C. P. analyzed. [3] Strip broken by accident while setting up the experiment.

TABLE 5

Effect of Citric Acid as Compared With Citric Acid + Ascorbic Acid + Iron on Peptic Activity, Using Freshly Cooked Egg-Albumen Strips as Substrate

Test	6% citric acid solution	HCl .02 N	H_2O	Ascorbic acid	$FeSO_4 \cdot 7 H_2O$	pH	Pepsin	Time required for strip to break	Decrease in breaking time; acceleration
	ml.	*ml.*	*ml.*	*mg.*	*mg.*		*mg.*	*min.*	*pct.*
A	8.1	1.0	20.9	None	None	2.24	100	63	.....
B	Duplicate of A							49	.....
							Average	56	38.4
C	8.1	0.5	21.4	21.9	34.5	2.24	100	70	.....
D	Duplicate of C							70.5	.....
							Average	70.2	22.9
E	None	18.7	11.3	None	None	2.24	100	92	.....
F	Duplicate of E							90	.....
							Average	91	Control

TABLE 6

Proteolytic Activity Present in Fresh Lemon Juice, Using Freshly Cooked Egg-Albumen Strips as Substrate [1]

Test	Lemon juice		6% citric acid solution	HCl .02 N	H_2O	pH	Minutes required for strips to break	
							Individual tests	Average
	ml.		*ml.*	*ml.*	*ml.*			
A	10	Raw	None	None	20	2.42	1130	.....
B	Duplicate of A			.....	.....	.....	1165	1147.5
C	10	Pasteurized [2]	None	None	20	2.42	>6300	.....
D	Duplicate of C			.....	.....	.....	>6300	.....
E	None	None	2.2—[3]	None	27.8+	2.43	>6300	.....
F	Duplicate of E			.....	.....	.....	>6300	.....
G	None	None	None	6.2	23.8	2.42	>6300	Control

[1] No pepsin added. [2] Pasteurized by heating in an open flask immersed in a hot water bath. Juice held at 86 to 91°C.(186.8 to 195.8°F.) for 30 minutes. [3] This amount of citric acid solution used to obtain approximately the same pH as in the other test mixes.

Maschmann and Helmert (1934) found that in the system papain-vitamin C-gelatin-peptone, the vitamin C retarded the action of the enzyme; while the substitution of vitamin C + iron for the vitamin C of that system resulted in a very considerable increase in activity.

The data (Tables 4 and 5) show that at a pH of about 2.2 in the system pepsin-lemon juice-ascorbic acid (vitamin C)-cooked egg albumen, the ascorbic acid retarded peptic action; and that the substitution of ascorbic acid + iron for the ascorbic acid resulted in a still further retardation of peptic action.

Therefore, in these two systems the effect of ascorbic alone is similar in that it retards enzymic action, but the effect of ascorbic acid + iron is quite different, accelerating in the one system and retarding in the other.

Tiselius and Ericksson-Quensel (1939) have offered two interesting explanations concerning the mechanism of the attack of pepsin upon the large molecules of which egg white is composed. Possibly the mechanism of the primary attack of pepsin upon cooked egg-albumen strips is in the nature of an orientation or of a rearrangement of the protein molecules which lessens the tenacity of the material and results in the breaking of the strip. It is probable that such a stage is reached before any considerable portion of the protein has been broken down to particles having a lower molecular weight; thus the mechanisms considered by Tiselius and Eriksson-Quensel are not fully applicable to the present work.

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SUMMARY AND CONCLUSIONS

The interdependent and combined effects of pH, lemon juice (also orange juice) or citric acid, on peptic activity were studied *in vitro*. Accelerations amounting to as much as 50 per cent in time reduction were observed under certain conditions of pH and substrate. Citric acid and some unidentified factor, present in fresh lemon juice, were responsible for the acceleration.

Lemon juice or a citric acid solution accelerates peptic action on sausage-casing substrate at pH 2.4 to about 1.5; but at pH about 1.5 to 1.1 either substance retards the action of pepsin.

Lemon juice or a citric acid solution accelerates the action of pepsin on freshly cooked egg albumen over the observed range: pH 2.4 to 1.1.

With *aged*, cooked egg albumen, lemon juice influences the action of pepsin in about the same manner as when sausage casing is used as substrate.

Lemon juice contains a relatively small amount of a proteolytic enzyme capable of the slow digestion of protein.

The experimental data indicate that the observed action of citrus juices on peptic activity was not due to ascorbic acid or to ascorbic acid + iron.

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NUTRITIONAL VALUE OF AUTOCLAVED AND RAW SOYBEANS, SUPPLANTED ONLY BY VITAMINS AND SALT MIXTURE[1]

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Morgan and Kern (1934) revealed that the nutritional value of meat proteins is lowered by heating. On the other hand, Osborne and Mendel (1917) and Vestel and Shrewsbury (1932) have shown that the protein of raw soybeans did not support appreciable growth, while normal growth resulted in rats when fed soybeans which had been previously exposed to heat. More recently Hayword, Steenbock, and Bohstedt (1936) demonstrated that the protein of expeller and hydraulic soybean oil meals prepared at high temperature were nutritionally superior to those prepared at low temperature. In their investigations all workers above showed that the protein of heated soybean was nutritionally superior to that of the raw bean in a diet in which the soybean was the sole source of protein in an otherwise balanced diet. The object of this investigation is to determine the relative nutritional values of raw and autoclaved soybean in a diet wherein the soybean is not only the sole source of protein (except for small amount of protein from yeast) but is, moreover, the only source of foodstuff in the diet.

EXPERIMENTAL PROCEDURE

The soybeans used were of the Manchu variety. They were pulverized to a consistency of flour in an electric grinder at the place of purchase. The schedule employed for autoclaving was that used by Hayword, Steenbock, and Bohstedt (1936) which involved heating the beans for one hour at 120°C.(248°F.) at 15 pounds pressure. The beans were placed in an autoclave in thin layers in shallow trays and exposed to this heat. The meal thus prepared was used without drying or regrinding. The diet given to all animals consisted of the following ingredients by weight:

Ground soybeans	93.7 per cent
Yeast (brewers)	5.0 per cent
$CaCo_3$	0.5 per cent
NaCl	0.5 per cent
Cod-liver oil	0.3 per cent

The soybean was analyzed for nitrogen and was calculated to contain 38 per cent protein. The animals, albino rats, were divided into two groups of 15 each; one group was fed the raw bean, the other was on an autoclaved bean diet. These rats came from three litters, were 32, 33, and 42 days old, and weighed 40 to 60 grams at the start. The initial and final weight for each animal was obtained, as well as weekly weighing, over the 33-day test period. The animals were housed individually in all-metal

[1] This paper was presented before the Southern California Chapter of the Society for Experimental Biology and Medicine, January 14, 1942.

cages equipped with sanitary drinking devices. The food was offered in double containers to prevent as much scattering as possible. The ad libitum type of feeding was used. A record was kept of the food consumption of each rat, every two or three days, for the 33-day test period.

The increase in weight of each animal was totaled and an average was determined. Similarly the total food consumed of each group was averaged. From the 38 per cent protein of soybean (plus two and one-half per cent protein of yeast) the average consumption of protein was computed. The nutritional value of the soybean was determined by the growth method of Osborne, Mendel, and Ferry (1919) as grams gained per gram of protein ingested; the results may be readily noted (Table 1).

TABLE 1

Nutritional Value of Soybean Determined by Growth Method

Type of diet	Average weight data			Average food consumption		Gain per gram of protein ingested
	Initial weight	Final weight	Average gained	Food ingested	Protein ingested	
				gm.	*gm.*	*gm.*
Raw soybean	46.80	77.73	30.93	147.50	56.20	.55
Autoclaved soybean	43.66	108.46	64.80	192.00	73.15	.89

SUMMARY AND CONCLUSION

In a diet wherein the soybean constituted the only source of foodstuff (a level of 40.5 per cent protein) steam autoclaving increased the nutritional value of the protein 62 per cent above the raw bean protein. Whereas most investigators find that the raw bean supports little or no growth at all, the raw soybean in this type of diet produced appreciable growth.

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CYSTINE CONTENT OF WHEAT AND ITS QUANTITATIVE DETERMINATION[1]

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Sulfur is essential for the growth and metabolism of the animal organism. It is a necessary part of hair, nails, hoofs, and other keratinous tissue as well as important enzymes. Inorganic sulfur cannot be utilized; the animal must receive its sulfur in amino acids. Consequently, much interest has developed concerning the cystine content of foods. The quantity occurring in many foods has been determined but little is known concerning the cystine content of wheat. Determinations which have been made of the total sulfur and nitrogen content of a number of varieties of wheat have shown significant variations in both, owing to variety and cultural treatments, Greaves and Bracken (1937). This study was undertaken to learn if the cystine sulfur constitutes a greater or lesser part of the sulfur and if the cystine content of the wheat[3] varies as does the total sulfur and nitrogen.

The literature on the numerous methods proposed for the quantitative determinations of cystine in biological products has been examined and two methods selected as the most promising: first, the method originally proposed by Sullivan (1926) and modified by Sullivan and Hess (1930) in which the reaction of cystine with betanaphthoquinone-4-sodium sulfanate was used; second, the one proposed by Folin and Looney (1922) and modified by Shinohara (1935-36a) based on the reaction of cystine with phospho-18-tungstic acid.

These methods were carefully checked for the following:

(1) Proportionality of color intensity with cystine concentration. This was done by comparing the color intensity obtained with pure solutions of cystine of varying concentration and the necessary reagents.

(2) Recovery of cystine after hydrolysis of mixtures of starch and gelatin, both of which are known to contain little or no cystine, to which a known quantity of cystine has been added.

(3) Determinations of cystine content of wheat, so as to check the consistency of the method.

The Sullivan method gives a depth of color which is variable and not proportional to the amount of cystine or cysteine present, even with pure solutions as shown by Lugg (1933) and Shinohara (1935). It also gives an orange-red color which too closely resembles the color of wheat hydroly-

[1] Contribution from the Department of Bacteriology and Biochemistry, Utah Agricultural Experiment Station. Report on Project 107, Purnell.

[2] Graduate assistant and research professor, respectively.

[3] The wheats analyzed were furnished by Professor A. F. Bracken and were grown under his direction on the Nephi Dry-Farm Substation.

sates for good results without decolorization. The color is hard to read in the colorimeter, since it does not give a clear line between the two halves of the field; hence, this method was discarded.

The phospho-18-tungstic acid reagent gives a blue color which is easy to read in the colorimeter. Lugg (1932) and Shinohara (1935) have shown that the depth of color is proportional to the cystine or cysteine concentration in pure solutions plus reagents if an excess of reagent is present.

The conditions in the determination of cystine in wheat are much different from those in pure solutions. A great number of products of hydrolysis are present, as well as an orange to yellow color, any or all of which may interfere. To approximate these conditions a mixture of gelatin and starch, both of which contain no cystine, was hydrolyzed with an added amount of .01 M. cystine, according to the procedure given by Sullivan and Hess (1937), using 20 per cent hydrochloric acid and 20 per cent titanous chloride, with subsequent precipitation of the titanium by changing to pH 6 with lithium hydroxide, filtration, and dilution to volume. When hydrolysis was made with either hydrochloric or sulfuric acid, as well as with and without titanium trichloride, a dark yellow hydrolysate was obtained. No significant difference was observed in the results obtained with these different methods of hydrolysis. The fact that the blank developed no color when only cystine plus hydrochloric acid and titanium trichloride were present indicates that the quantity of mercuric chloride added is sufficient to combine with all of the cystine present.

In order to learn whether the reagents interfere, the regular amounts of all were added to one aliquot of sample; to another, twice the phosphotungstic acid; to a third, twice the sulfite; and to a fourth, no sulfite. When twice the color reagent was used, slightly higher recoveries were obtained. Quite large variations in the amount of phosphotungstic acid reagent added is permissible so long as sufficient is present, without appreciably affecting the color development. Thus, twice the recommended amount gave lower percentages of recovery than did the recommended amount (74 and 70 per cent for the regular amount and 64 and 58 per cent for double amounts of sulfite). Sulfite reduces the reagent more slowly than does cystine; therefore the color developed within the time allowed for cystine color development will not have an appreciable effect on the results. For best results the quantities of all reagents should be accurately measured.

The various reagents were tested individually and in combination for their effect on the phosphotungstic acid reagent with the following results:

(1) Mercuric chloride + reagent.....no color.
(2) Sulfite + buffer + reagent.....slight color.
(deeper color on addition of excess reagent)
(3) Sulfite + buffer + hydrochloric acid + titanium trichloride + lithium hydroxide.....very slight color.
(4) The same + mercuric chloride.....very slight color.
(5) Hydrochloric acid + titanium trichloride.....no color.

The addition of bromthymol blue to the standard to compensate for the yellow color of the hydrolysates gives good results. With bromthymol blue only one per cent error in recovery of cystine occurred, whereas with

determinations on the same sample without bromthymol blue, six, four, and seven per cent errors occurred.

Decolorization and extraction with ether were tried, with errors of 28 and 23 per cent, respectively, indicating that errors owing to absorption in decolorization or extra manipulation are greater than those owing to any extraneous color or reducers present.

Both HCl and H_2SO_4 were tried for hydrolysis, and H_2SO_4 was found to be much better, since HCl gave dark red hydrolysates which could not be used without drastic decolorization, whereas H_2SO_4 produced only a light yellow-colored hydrolysate. Titanium chloride was also found to give a dark hydrolysate which could not be used. Sullivan and Hess (1937) state that the use of $TiCl_3$ decreases the "humus" formation, as well as shortens the time required for hydrolysis from 18 to two hours; however, this was not found to be the case in our experiments. Its use necessitated more manipulation and caused difficulties in filtering. Since the pH must be raised to six for precipitation of the $TiCl_3$, some loss of cystine may also occur.

A large number of determinations were made on several varieties of wheat without the addition of cystine. If duplicate determinations were made at the same time, a fair agreement was obtained; but if made on different days or even at different times on the same day, wide variations occurred in the cystine content found. The standards were checked and found not to vary. Decolorization was again tried, but considerably lower and more inconsistent results were obtained than when it was not used.

Extraction with ether and chloroform to remove pyruvic acid, levulinic acid, etc., was also tried, as suggested by Lugg (1932) and Shinohara (1935). Neither procedure gave as good results as without the extraction, and none of the color was extracted by them. In fact, ether seemed to cause even greater interference.

One difficulty encountered was the rapid increase in color over a relatively long period of time, so that consistent readings were difficult to obtain, Shinohara (1935-1936b). The color intensity would increase appreciably, even during the time required to make the reading. Lugg (1932) found that color development may vary with time, concentration of cystine, concentration and type of extraneous reducers present, pH, salt concentration and type, and order of addition of reagents. Shinohara (1935-36a) and Lugg (1932) recommend a pH between 4.7 and 5.4 as the range where the time for and intensity of color development are constant. Below this range it is too slow and incomplete, whereas at higher pH values the color resulting from extraneous reducers develops too fast and then fades too rapidly. Shinohara suggests a buffer (three c.c. 2 M. acetic acid and 10 c.c. 2 M. sodium acetate) to maintain this constant pH. The standards used in this work were .2 molar in H_2So_4. To give test solutions of this same acidity, only half the amount of NaOH necessary to neutralize the hydrolysate to pH 3.5 was added after hydrolysis. The buffer mixture necessary to give the desired pH of 4.7 was found to be one c.c. of acetic acid and 10 c.c. of sodium acetate. Two c.c. of 1 M. NaOH or LiOH were added to each 10 c.c. of the standard or hydrolysate to neutralize the excess acidity.

During hydrolysis of the samples, the sides of the flasks were carefully washed with a small quantity of distilled water from a wash bottle at four and 12 hours after the hydrolysis was started. When this was not done, a layer of charred material collected on the sides of the flasks probably causing considerable variation.

Exactly the same time was allowed between addition of the color reagent and comparison in the colorimeter for both the test solution and the blank. If there was a difference of a few minutes more or less between the time allowed for color development in the blank and that for the test solution, correspondingly lower or higher results were obtained.

After these modifications were made in the method, higher and more consistent results were obtained; the results of determinations before and after the modifications were used are given (Table 1).

TABLE 1

Values in Molar Concentration of Cystine in Hydrolysates Obtained Before and After Modifications Were Made in Method

	Molar concentration (x 10^{-4})					
Sample No.	1	2	3	4	5	6
Before	1.86	1.04	.....	.....	1.81	.....
	1.41	1.75	.....	.....	2.40	.....
	2.39	0.93	.....	.....	1.75	.....
	1.49	1.64	.....	.....	1.89	1.64
	1.32	1.39	.....	1.58	1.70	1.64
	1.87	1.68	.....	1.51	1.63	1.27
	1.83	1.25	1.71	1.79	1.81	2.06
	1.57	1.68	1.75	1.59	1.69	1.40
	1.75	1.69	1.71	1.51	1.49	1.74
	1.83	1.70	1.72	1.65	1.93	1.96
After	2.28	2.27	2.32	2.43	2.09	2.39
	2.34	2.20	2.18	2.42	2.25	2.39
	2.32	2.02	2.48	2.34	2.03	2.58
	2.08	2.22	2.39—	2.42	2.33	3.10
	2.40	2.23	2.07	2.20	.....	.....
	2.37	2.05	2.30	2.24	.....	.....
	2.38	1.96	2.42	2.20	.....	.....

EXPERIMENTAL PROCEDURE

The procedure followed in the analyses of the wheats was as follows:

(1) Three-gram samples of the finely divided wheat grown and prepared for analysis as described by Greaves and Bracken (1937) were weighed and transferred to 200-c.c., round-bottomed flasks, 15 c.c. of 6 N. H_2SO_4 added to each, and the flasks fitted with return condensers. They were then hydrolyzed for 14 to 16 hours on an oil bath at 115 to 125°C. (239 to 257°F.).

(2) The sides of the flasks were washed down twice during hydrolysis, usually after four and 12 hours.

(3) Nine c.c. of 5 N. NaOH were then added to neutralize acidity to about .2 N. in sulfuric acid, the hydrolysate filtered through two sheets of ordinary filter paper, and diluted to 200 c.c. at 20°C.(68°F.).

(4) Aliquots of 10 c.c. were transferred to each of two 50-c.c. volumetric flasks (or usually five-c.c. to 25-c.c. flasks). To each flask 10 c.c. of 2 M. sodium acetate, one c.c. of 2 M. acetic acid, and two c.c. of 1 M. sodium hydroxide, or lithium hydroxide, were added. Usually four samples were run at a time and treated as follows: three c.c. of 1 M. sodium bisulfite were next added to each of the first two flasks and the time noted. After one minute, four c.c. of the color reagent were added to the first flask, and three c.c. of .1 M. mercuric chloride to the second (the first is the test solution, giving the total color; and the second is the blank, giving only the color owing to extraneous reducers, since the cystine was combined with the mercuric chloride). After two minutes the sulfite was added to the next two; and then, after another minute, the color reagent was added to one and the mercuric chloride was added to the other. This procedure was carried out in this manner for all four samples. Twelve minutes after the addition of the reagent to each flask, the reading or comparison was made with a suitable standard. This procedure results in the readings being made at three-minute intervals. Each blank was thus read exactly 20 minutes after the addition of the reagent, just as was each test solution. If the test solutions were allowed to stand longer before comparison, the blanks were also left the same length of time.

(5) Two or more appropriate standards were prepared at the same time and in the same manner as the test solution, except that an appropriate amount of bromthymol blue was added to each standard. The color developed quickly in the standards, but only slowly in the test solution and blanks containing the extraneous reducers. The standards may be used for three or four hours without much error if they are kept in a cold, dark place.

(6) A Bausch and Lomb colorimeter was used. Ten-c.c. portions of the colored solutions were used in the cups.

(7) The determinations were made as soon after hydrolysis as possible, since prolonged standing seems to result in some loss of cystine.

RESULTS

The percentages of cystine found ranged from .338 to .405 per cent for the samples of wheat analyzed, with an average of .364 per cent (Tables 2 and 3). In spring wheats the range was from .346 to .405 per cent, with an average of .372; and for winter wheats a range from .338 to .385 per cent, with an average of .361. These results show a greater average per cent of cystine in spring wheats than in winter wheats, but there is an overlapping of results in spring and winter wheat, hence this difference may not be significant. However, Greaves and Bracken (1937) did find a significant difference in the total sulfur and nitrogen.

The results were analyzed statistically to learn whether or not there was a difference between variety means. The F-value approached significance but did not reach the five per cent point indicating essentially as much variation between different determinations of the same variety as between variety means.

Comparisons made with data on the total sulfur and nitrogen disclose several interesting things. In general, variations in sulfur content are

TABLE 2

Percentages of Cystine, Cystine-Sulfur, and Cystine Sulfur-Total Sulfur Ratio of Five Varieties of Spring Wheats Grown Under Similar Cultural Conditions and on the Same Soil

Variety	Cystine	Cystine-sulfur	Cystine sulfur-total sulfur ratio [1]
	pct.	*pct.*	
Hard Federation	.405	.108	.492
Baart	.370	.099	.471
Kubanka	.379	.101	.505
Marquis	.346	.092	.511
Chul	.361	.096	.534
Average	.372	.099	.502

[1] Calculated from data given by Greaves and Bracken (1937).

TABLE 3

Percentages of Cystine, Cystine-Sulfur, and Cystine Sulfur-Total Sulfur Ratio for 12 Varieties of Winter Wheats Grown Under Similar Conditions and on the Same Soil

Variety	Cystine	Cystine-sulfur	Cystine sulfur-total sulfur ratio [1]
	pct.	*pct.*	
Turkey	.385	.103	.491
Kharkov	.371	.099	.521
Utac	.385	.102	.550
Sevier 34	.371	.099	.550
Kharmont	.341	.091	.506
Montana 36	.352	.094	.523
Kofod	.364	.097	.538
Tenmarq	.370	.099	.583
Alton	.346	.092	.574
Newturk	.338	.090	.600
Regal	.346	.092	.541
Kharkov-Hayes 2	.370	.099	.660
Average	.361	.096	.553

[1] Calculated from data given by Greaves and Bracken (1937).

TABLE 4

Percentages of Cystine, Cystine-Sulfur, and Cystine Sulfur-Total Sulfur Ratio for Kanred Winter Wheat Grown Under Four Different Cultural Conditions

Treatment	Cystine	Cystine-sulfur	Cystine sulfur-total sulfur ratio [1]
	pct.	*pct.*	
Peas plowed under when in pod	.365	.097	.511
Peas plowed under when 6″ high	.338	.090	.500
Wheat plowed under when 6″ high	.340	.090	.563
All straw returned to land and burned	.379	.101	.562
Average	.356	.095	.534

[1] Calculated from data given by Greaves and Bracken (1937).

accompanied by corresponding variations in cystine content. Though this does not hold in all cases, it seems to be the general trend. The higher average percentage of cystine in spring wheats, as compared with that for winter wheats, is paralleled by a correspondingly higher average value for total sulfur and nitrogen. The variations between the lowest and highest sulfur value is about 46 per cent, whereas that for the cystine value is only 20 per cent, so not as wide a range was obtained in percentages of cystine as in those for sulfur. The percentage difference between the lowest and highest nitrogen values is about 31 per cent, which is intermediate between the two.

It will be observed that the cystine-sulfur, as found, constitutes about 50 per cent of the total sulfur, the average being 54.4 per cent and the percentage ranging from 47.1 to 66 per cent as shown by the cystine sulfur-total sulfur ratio. This gives a range of 21 per cent between the highest and lowest values. It is also interesting to note that, in general, as the total sulfur and cystine content increases there is a decrease in the percentage of the total sulfur represented by cystine. This is also seen to be the case with the nitrogen-sulfur ratio as given by Greaves and Bracken (1937). Thus, Hard Federation has a cystine content of .405 per cent and total-sulfur content of .22 per cent (the highest), and a cystine sulfur-total sulfur ratio of only .492, or 49.2 per cent; whereas Newturk, with a cystine content of .338 per cent and sulfur content of .15 per cent (the lowest), has a cystine sulfur-total sulfur ratio of 6.0, or 60 per cent. The nitrogen-sulfur ratios for these same two varieties is 13.9 and 16, respectively. Calculation of the coefficients of correlation of cystine to total sulfur and cystine to nitrogen gave values of .83 and .75, respectively, as compared with a correlation coefficient of .81 for total sulfur and nitrogen as given by Greaves and Bracken (1937).

The four samples of Kanred wheat representing different cultural treatments seemed to follow the same general trends discussed above. The number of samples is too small to be statistically analyzed with any degree of accuracy.

Csonka (1937) found variations in the cystine content between varieties of wheat of .27, .17, and .15 per cent, respectively, for Marquis, Tenmarq, and Fulhio, and corresponding variations in nitrogen of 3.31, 2.58, and 2.34 per cent, respectively.

Greaves, Bracken, and Hirst (1940) found significant variations in the mineral content between varieties of wheat and also within the same variety owing to varied cultural treatment.

Sullivan and Howe (1929), Guillemont and Schnell (1933), and Greaves and Bracken (1937) suggest that all or nearly all of the sulfur of wheat is in the form of organic sulfur.

CONCLUSIONS

The F-value obtained from a statistical analysis of the data approached significance but did not reach the five-per cent point indicating essentially as much variation between different determinations of the same variety as between variety means. Some winter wheat carries as much cystine-sulfur

as does some spring wheat, but there is a slightly higher percentage of cystine in winter wheats. Variation in cultural treatments apparently cause a variation in the cystine content, though the results here are not such as to warrant a definite statement to that effect.

The coefficients of correlation found for sulfur and cystine and nitrogen and cystine are highly significant, as was found to be the case with nitrogen and sulfur by Greaves and Bracken (1937). This leads to the conclusion that, as the total sulfur and nitrogen vary, a corresponding variation in the cystine content should be found.

The fact that the cystine sulfur-total sulfur ratio decreases with an increase in the percentage of total sulfur and cystine may mean one of two things; either the errors of determination of cystine become greater with an increase in the amount present in the wheat, or the relative amount of sulfur present in the form of cystine decreases with increasing total sulfur content. If this latter conclusion were true, it might indicate either a greater proportion of inorganic sulfur or a greater proportion of the organic sulfur in some other form than cystine, which is not determined by this method. Present available information does not indicate definitely which of these possibilities may be correct.

The data lead to the conclusion that an average of about 50 per cent of the total sulfur of wheat is in the form of cystine, and that this percentage of cystine may be affected by both variety and cultural treatment.

These conclusions are, in general, in harmony with the findings of other investigators, as referred to above, both for wheat and for other biological materials.

Wheat compares favorably with other cereals used as food as a source of cystine in the diet. Soybeans are somewhat higher in cystine than wheat (also higher in protein). Barley and oats rank close to wheat as sources. Oat gliadin is very high in cystine. More work needs to be done, however, before any definite conclusions can be reached as to the relative value of these foods as sources of cystine in the diet.

SUMMARY

The various methods for the determination of cystine were reviewed and two selected which seemed to fulfill most nearly the requirements for a good method.

These were checked and the colorimetric method proposed by Folin and Looney (1922) and modified by Shinohara (1935) was chosen. A number of modifications were made by the authors and this method used to analyze wheat.

Seventeen varieties of wheat grown on the same soil and under the same conditions were analyzed and found to contain an average of .364 per cent of cystine. This corresponds to .097 per cent of cystine-sulfur and represents 54.4 per cent of the average total sulfur. A probable variation in cystine was found between varieties as well as between wheats grown under varying cultural conditions, and a positive correlation was found between cystine and sulfur as well as between cystine and nitrogen.

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YOUNG PIGS AS TEST ANIMALS FOR STAPHYLOCOCCUS ENTEROTOXIN

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The methods for determining which of the Staphylococci are able to produce substances causing gastrointestinal disturbances in man rest mainly upon the reaction of animals to the feeding or injection of a filtrate of the suspected culture. Much work has been done to increase the reliability of such animal tests by the use of various species of animals and through administration of culture filtrates by different routes. Nevertheless, the literature on this subject shows that no one experimental animal is accepted for such tests by a majority of workers. It is the purpose of this paper to demonstrate that young pigs are sensitive and reliable animals for the testing of the presence of enterotoxin in Staphylococcus filtrates.

Early in the history of Staphylococcus food poisoning, monkeys were used as test animals by Jordan and McBroom (1931-1932) and have since been used by a number of other workers: Woolpert and Dack (1933), Davison and Dack (1939), and Davison, Dack, and Cary (1938). However, Minett (1938) and Grubb (1938) did not find the monkey to be a satisfactory test animal for Staphylococcus enterotoxin.

The use of kittens and cats is favored by certain workers: Dolman and Wilson (1938, 1940), Kupchik (1937), Phatak and Pentler (1940), Bayliss (1940), and Hammon (1941). On the other hand, Davison and Dack (1939) report that consistent reactions were not obtained with kittens. The observation made by Hammon (1941) and Rigdon (1938) that vomiting is induced by the injection of uninoculated filtrate is a most important objection to the use of kittens.

Dogs are rarely used in enterotoxin tests. Minett (1938) stated that dogs which had been fed culture filtrates did not vomit. Hammon (1941) found that puppies vomited when given intraperitoneal injections of filtrates, but that the reaction was not always due to enterotoxin.

Since the domestic pig is stated to have a well-developed vomiting reflex and vomits readily, Wooldridge (1934), a series of tests were carried out to determine the susceptibility of young pigs to Staphylococcus enterotoxin.

EXPERIMENTAL PROCEDURE

Pregnant sows were obtained several weeks before the birth of the young. A mixed ration was fed dry and water was supplied separately to exclude any possibility of the growth of Staphylococci in the food supplied to the mother pig. This precaution was taken to avoid passive immunization of the unborn pigs to the enterotoxin formed by Staphylococci.

In these tests, nine litters containing a total of 57 young pigs of mixed breeds were used. The Hampshire crosses were preferred as the young pigs were smaller and could be handled with ease to a greater age than was the case with heavier breeds, such as the Chester White.

In the tests with the first four litters, repeated injections were made of filtrates from a strain of *Staphylococcus aureus* which had caused illness in humans and filtrates of which had produced vomiting in monkeys. With the last five litters, the cultures used were suspected strains of various origin.

The cocci were grown in Meleney and Chatfield's (1931) broth for 18 to 24 hours and an inoculation of two ml. of this young culture was made on semisolid veal infusion agar (fresh veal infusion with .5 per cent peptone and .5 per cent agar added) contained in Kolle flasks. The flasks were incubated for three or four days, or in a few cases for two or two and one-half days in an atmosphere of 25 per cent CO_2 and 75 per cent air. The medium was then poured on filter paper to remove the agar, centrifuged, heated in a boiling water bath to destroy lethal, hemolytic, and dermonecrotic toxins, and filtered through a Seitz filter. The filtrates were held in the refrigerator until used, generally within a few days after preparation.

For control tests two types of material were prepared. The first was active filtrate which had been autoclaved for 45 minutes at 15 pounds steam pressure to destroy the enterotoxin. The second was uninoculated infusion broth which had been passed through filter paper to remove the agar and then through a Seitz filter to remove contaminating bacteria.

In looking for a suitable route for the administration of suspected filtrates to young pigs, oral feeding was tried first. A litter of pigs, eight days old and weighing 2.10 to 2.50 kg., were used. Three pigs were fed filtrates, prepared as described, using a strain of *Staphylococcus aureus* known to be capable of producing an enterotoxic substance. The amounts fed varied from 12 to 35 ml. As controls three pigs were fed with 25 ml. of the above filtrate which had been autoclaved at 15 pounds pressure for 30 minutes. The pigs were then observed for a period of five hours. All appeared to react alike. All the pigs including the controls were restless and irritable between periods of drowsiness, but no vomiting occurred.

As oral administration did not appear to be satisfactory, an intraperitoneal injection was tried on one pig. This pig vomited in two hours and a number of times thereafter at short intervals. Following further preliminary tests on a number of pigs, intraperitoneal injection was adopted as the route of administration.

After a number of trials were made to determine the amount of filtrate necessary to cause a reaction as well as the volume of injected liquid which could be tolerated by the pigs, a dose of three to four ml. per kg. was found to be suitable. This amount could be readily administered, caused only temporary discomfort to the animals, and resulted in a large percentage of reactions.

The symptoms observed after the injection of filtrates containing enterotoxin were as follows. Signs of discomfort were shown by the pigs soon after injection, and periods of restlessness and irritability alternated with periods of drowsiness. Vomiting was preceded by convulsive movements of the abdominal wall which increased in severity to the time when vomiting took place. Reactions were called positive only when vomiting did occur. In most cases vomiting resulted in 30 to 90 minutes after the

injection of enterotoxin, though it was sometimes delayed to nearly three hours.

As control tests, injections were made of toxic filtrates, autoclaved for 30 to 45 minutes at 15 pounds steam pressure, as well as uninoculated filtered medium. Dosages of three to 4.4 ml. per kg. were given. No positive reaction occurred in any control test.

In comparative tests with monkeys and pigs, the monkeys failed to react to a filtrate which contained sufficient enterotoxin to produce vomiting in pigs. Susceptible monkeys weighing 2.7 to 3.6 kg. were used. Two were fed 50 ml. of filtrate, and three were injected intraperitoneally with dosages of 2.5 to 6.0 ml. of filtrate per kg. None of the monkeys vomited, while all of the pigs injected intraperitoneally with 2.1 to 4.3 ml. of

TABLE 1

Reaction of Young Pigs to Repeated Intraperitoneal Injections of Control and Toxic Filtrates, Second Litter

Age	Pig No. 1	Pig No. 2	Pig No. 3	Pig No. 4	Pig No. 5	Pig No. 6	Pig No. 7
days							
	Control [1]	Control [1]	Control [1]				
7	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
	Control [1]	Control [1]	Control [1]				
12	n.r.	n.r.	n.r.	60	40	55	70
	Control [1]	Control [1]	Control [1]				
15	n.r.	n.r.	n.r.	n.r.	70	n.r.	50
	Control [1]	Control [1]	Control [1]				
19	n.r.	n.r.	n.r.	70	....	50	35
27	n.r.	85 [2]	90	60	....	70	45
34	n.r.	n.r.	65	60	....	n.r.	50

[1] Filtrate autoclaved for 45 min. at 121.1° (250° F.); n.r. = negative reaction; dosage = three ml. filtrate per kg. [2] Reaction times are given in minutes.

filtrate per kg. vomited. These results indicate that pigs show greater sensitivity to enterotoxin than monkeys.

The intervals at which injections were made necessarily differed from test to test. During the experiments on the first four litters, when the reliability of the reaction of young pigs to enterotoxin was being determined, injections were given about once a week. In this way, it was possible to note the effect of repeated injections of enterotoxin containing filtrates and of control filtrates on the sensitivity of the animals, as well as the specificity of the test.

Detailed data of the age at the time of injection and reaction at each injection are given for only one litter. This litter was typical of the results obtained on the other eight litters and no useful purpose is served by including information other than summary data on all of the pigs.

Results obtained on repeated intraperitoneal injections of control and toxic filtrates are given (Table 1). In this group of pigs a uniform dose of three ml. per kg. was used for each of the six injections. The age at the time of injection varied from seven to 34 days while the weight increased

from 2.02 to 2.51 kg. at the time of the first injections to 3.18 to 5.30 kg. at the last injections. From these and later results it appears that neither age nor weight has any marked effect on the reaction of the pigs.

Pigs 1, 2, and 3 received four successive injections of autoclaved filtrate and did not react to any of these injections. On the fifth and sixth injections they were given an active filtrate. Pig 1 failed to react at all, and thus cannot be used in the control animals, since it was not shown to be a reactor. Pig 2 reacted to one of the two, and Pig 3 to both of the active filtrates.

The filtrate used in the first injections of Pigs 4 to 7 gave no reaction in any of the pigs owing to the failure of the culture to form sufficient enterotoxin. As many workers have observed, strains which normally form enterotoxin in quantities, may, without any change in cultural methods,

TABLE 2

Reaction of Young Pigs to Repeated Intraperitoneal Injections

Number of injections of enterotoxin-containing filtrates	Number of pigs injected	Number of pigs reacting to each injection
One	57	53 (93 per cent)
Two	29	24 (83 per cent)
Three	17	15 (88 per cent)
Four	11	4 (36 per cent)
Five	3	1 (33 per cent)

sometimes produce very little or no enterotoxin. The filtrates prepared for the next five injections of Pigs 4 to 7 induced reactions in a majority of the animals.

Pig 4 reacted to four out of these five injections; Pig 5 reacted to the first two and then died; Pig 6 reacted to three of the five; and Pig 7 reacted to all five injections. These and similar results obtained on other litters indicate that young pigs may be used for repeated injections.

A summary of the number of pigs reacting to each injection (Table 2) shows the number of injections of enterotoxin-containing filtrates, the number of pigs receiving those injections, and the number of pigs reacting to each injection. The data are presented in this form to indicate that pigs may be used for several tests without a marked decrease in sensitivity.

Of 57 pigs injected with filtrates containing enterotoxin, 53, or 93 per cent, reacted. These results indicate the young pig to be a reliable animal for enterotoxin tests. When given two and three injections of active filtrate, the percentages reacting were 83 and 88 per cent, respectively. Thus, sensitivity is largely retained for three exposures to enterotoxin. On the fourth and fifth injections, only a third of the pigs continued to react. However, the number of animals receiving three or more injections of filtrates containing enterotoxin was too limited to permit definite conclusions on the number of times an animal may be used.

The number of control tests and the types of material injected are given (Table 3), the results given are for susceptible animals only, and include no data on such animals as Pig 1 in Table 1 which, after four control injections, failed to react when given active filtrate in the fifth

and sixth injections. Dosages were three and four ml. per kg. No pigs vomited when injected with autoclaved filtrate, with uninoculated filtered medium, or with filtrates from nontoxin-forming cultures of Staphylococci. These latter cultures were part of a group of strains of unknown toxigenic ability. In this group, the greater number did not form enterotoxin.

TABLE 3

Control Tests—Intraperitoneal Injection of Enterotoxin-Free Liquid Into Young Pigs

Injected material	Number of injections	Number of reactions
Autoclaved filtrates	13	None
Uninoculated medium	8	None
Nontoxin-forming strains	30	None

In order to reach some conclusion regarding the number of pigs required for a test and what degree of accuracy could be expected of the result, the data for the first four litters of pigs were arranged in all possible combinations of twos and threes. This was done, of course, only for those tests in which one or more pigs reacted. These combinations included the non-reactors, since there is the possibility that one or more pigs in a litter may be resistant to Staphylococcus enterotoxin. Furthermore,

TABLE 4

Reactions of Pigs in Random Combinations of Two and Three Pigs

Combinations of two	Number of combinations
No pig reacting	14
One pig reacting	60
Two pigs reacting	92
	166

Chance of negative result with positive filtrate 14/166 or 8.4 in 100.

Combinations of three	Number of combinations
No pig reacting	6
One pig reacting	40
Two pigs reacting	88
Three pigs reacting	97
	231

Chance of negative result with positive filtrate 6/231 or 2.6 in 100.

these combinations of animals within each litter were made without regard for the number of injections which the animals had already received. Thus the probability figures are based on data similar to those obtained under actual conditions of testing in which the animals are used repeatedly (Table 4).

With combinations of two pigs, 166 arrangements were possible. Of these 166 arrangements, in only 14 did neither pig react. Thus, if two pigs were used for testing a filtrate, in 8.4 cases out of 100 can a negative result be expected through sampling error, that is, through unfortunate

selection of the two test animals. When three pigs are taken for a test, the chance of a negative result being obtained by sampling error is greatly reduced, and is then 2.6 chances out of 100. This indicates that if three animals are used in a test and one or more reacts, the results have considerable significance statistically. These figures are presented to aid other workers in deciding how many animals to use, and indicate the accuracy which may be expected in their tests.

DISCUSSION

In contrast with other animals used for enterotoxin tests, young pigs are cheap and obtainable any time of the year. The gestation period of the pig is four months and the estrus cycle three weeks. Moreover, six to 10 young are born in a litter. Very little special attention is required by the animals other than feeding a complete dry ration once a day and supplying them with water.

CONCLUSIONS

Intraperitoneal injections into suckling pigs of Staphylococcus filtrates containing enterotoxin induced vomiting in 53 of the 57 pigs tested. The specificity of this response was shown by failure of the animals to react when injected with autoclaved filtrates from enterotoxic strains, filtrates from non-enterotoxic strains, or filtered, uninoculated medium.

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METHYL BROMIDE AS A FUMIGANT FOR FOODS

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The efficiency of methyl bromide as a fumigant for the eradication of certain insect pests was discovered by Le Goupil (1932) in 1931, when using it as a fire retardant in ethylene oxide mixtures. Pioneer work with methyl bromide in this country has been carried out by Mackie (1938) and Hawkins (1938, 1939) and their associates. Thousands of tons of many types of foodstuffs are now being fumigated with methyl bromide annually.

Methyl bromide is a colorless organic liquid, having the formula CH_3Br, with a boiling point at 4.6°C.(40°F.), so that at ordinary room temperature it is a gas. The specific gravity of the gas is 3.27 (air = 1). The inflammable limits are within a very narrow range, 13.5 to 14.5 per cent by volume. The methyl bromide now supplied in commercial quantities is of high purity, being more than 99 per cent pure. Methyl bromide has a slight aromatic odor in high concentrations. At low concentrations, which may still be toxic, the gas has no odor.

In this experimental study the problem of the consumer hazard of methyl bromide has been approached from two angles: first, investigation of foodstuffs, with determination of the rate of release of methyl bromide and the amount of residual bromide following fumigation; and second, the physiological and pathological changes induced by consumption of methyl bromide-treated foodstuffs as shown in experimental animals.

The results of analysis for total bromides in various classes of fumigated foodstuffs (Table 1) are given as milligrams of bromide per 100 grams of sample. Examination of this table will show that in general fresh fruits and vegetables, dried fruits, and whole grains, absorb rather small amounts of the fumigant, while the bromide content drops rapidly during the first 48 hours after fumigation. On the other hand, milled grain, cheese, and nuts absorb much greater quantities of methyl bromide with considerable quantities retained longer than 48 hours.

The results indicate that the amount of methyl bromide (determined as bromide) absorbed by the produce during fumigation is several times the normal bromide content. In most cases the fumigated material showed a drop in bromides after aeration. Dried fruits, fresh fruits, and vegetables absorbed minor quantities of the fumigant. The foodstuffs which absorb greater amounts of the fumigant include milled grains, cheese, nuts, and nut meats. The adsorptive capacity of milled grains is due in part to the great surface area exposed, while oily and fatty foods absorb large quantities of methyl bromide because of its solubility in fats.

FEEDING EXPERIMENTS

Feeding experiments utilizing diets containing moderate and excessive amounts of organic and inorganic bromides following fumigation with methyl bromide were carried out with young white rats and rabbits.

The rat-feeding experiments were made in three groups: the first was a preliminary eight-week feeding test; the second continued 16 weeks; and the third group of rats was fed various fumigated diets for 20 weeks. The rabbit-feeding experiments were carried on for 52 weeks. Studies were also made of the acute effects on rabbits of single doses of methyl bromide fed in olive oil solution.

Rat-Feeding Experiments (Table 2 and Fig. 1): Feeding experiments with rats, over a period of 20 weeks, show that when excessive (620 to 529 mg. Br per 100 gm.) amounts of organic and inorganic bromides are

TABLE 1

Bromide Content of Fruits and Vegetables After Laboratory Fumigation With CH_3Br [1]

Sample	Mg. Br/100 gm. sample				Dosage CH_3Br at atmospheric pressure 20 to 25°C. (68 to 77°F.)
	Before fumigation (control)	Immediately after fumigation	24 hours after fumigation	48 hours after fumigation	
White potatoes					
Peel	2.58	4.22	3.66	3.02	2 pounds CH_3Br/1,000 cubic feet for 2 hours
Pulp	0.79	1.28	1.29	1.00	
Sweet potatoes					
Peel	1.66	3.16	3.20	3.16	Do.
Pulp	0.55	0.99	0.98	0.90	Do.
Green beans	0.54	7.22	4.20	4.08	Do.
Tomatoes	Trace	1.26	1.11	0.91	Do.
Eggplant	0.10	2.39	2.11	1.72	Do.
Onions	Trace	0.80	0.62	0.61	Do.
Carrots	Trace	2.59	1.23	1.20	Do.
Beets	0.83	3.65	3.83	3.16	Do.
Turnips	None	2.43	2.09	1.58	Do.
Apples (fresh)	None	0.30	0.31	0.27	Do.
Pears (fresh)	None	0.28	Trace	None	Do.
Corn (whole grain)	None	0.70	Trace	Trace	2 pounds CH_3Br/1,000 cubic feet for 24 hours
Corn meal (white)	None	5.82	3.26	2.90	Do.
Wheat (whole grain)	None	0.95	Trace	Trace	Do.
Flour (white)	None	11.10	4.54	4.26	Do.
Flour (whole wheat)	None	7.44	6.35	5.18	Do.
Oats (whole grain)	Trace	4.00	2.96	2.60	Do.
Rolled oats	Trace	17.24	14.68	13.02	Do.
Barley (whole grain)	Trace	0.52	Trace	Trace	Do.
Rice (brown, whole grain)	None	1.58	1.38	1.22	Do.
Raisins (seedless)	None	0.36	0.28	0.26	Do.
Peaches (dried)	1.44	2.31	1.86	1.60	Do.
Apricots (dried)	0.90	1.89	.....	1.18	Do.
Prunes (processed)	None	0.27	Trace	Trace	Do.
Pecans (whole nut)	None	7.00	7.00	6.90	Do.
Peanuts (whole nut, unroasted)	None	5.04	5.00	5.00	Do.
Pecan nut meats	None	21.50	13.14	12.66	Do.
English walnut meats	None	11.50	8.70	7.44	Do.
Cashew nut meats (unroasted)	None	22.92	15.20	14.88	Do.
Peanuts (shelled, unroasted)	None	7.88	5.46	4.74	Do.
Cheese (yellow American)	0.85	8.01	8.10	7.65	Do.

[1] Values are not corrected for moisture content of sample. Results are average of three or more determinations on samples from same lot of material. Parts CH_3Br per million (mg. CH_3Br/kilo of sample) may be computed as follows:

$$12 \times \left[\left(\begin{matrix} \text{Br content 24 hours after fumigation} \\ \text{(mg./100 gm.)} \end{matrix} \right) - \left(\begin{matrix} \text{Br content before fumigation} \\ \text{(mg./100 gm.)} \end{matrix} \right) \right] = CH_3Br, \text{ p.p.m.}$$

TABLE 2

Rat-Feeding Tests, 20 Weeks

Run No.	Number of rats on test	CH_3Br content of food (p.p.m.)[1]	Treatment of food and feeding program [2]	Results
1 (control)	36	None (unfumigated)	Rat pellets *(ad lib.)* unfumigated.	Excellent weight gains (1 death). General condition good.[4]
2	36	(Time fed after fumigation) Rat pellets: At once, 284 In 24 hours, 253 Average, 270	Rat pellets *(ad lib.)*, fumigated for 24 hours at 3 pounds CH_3Br /1,000 cubic feet. Fed immediately after fumigation.	Good weight gains (4 deaths). General condition good. Reproduction normal. Average weight gains, 10 per cent below controls. No significant pathology.[4]
3	36	Rat pellets: In 24 hours, 253 In 48 hours, 234 Average,245	Same diet as for Run 2 except food aerated for 24 hours before feeding.	Good weight gains (2 deaths). General condition good. Reproduction normal. Average weight gains, 11 per cent below controls. No significant pathology.[4]
4	36	Rat pellets: At once, 7,375 In 24 hours, 6,285 Average, 6,830	Rat pellets *(ad lib.)*, fumigated for 24 hours with undiluted CH_3Br gas. Fed immediately after fumigation.	Condition poor (15 deaths). Weight gains slight, 30 per cent below controls. No reproduction. Activity reduced. Little significant histopathology.[4]
5 (control)	36	None (unfumigated)	⅓ diet rat pellets, remainder made up of 3 of following articles: sweet potatoes, green beans, dried peaches, cheese, peanuts. Pellets and others all unfumigated.	Condition excellent (4 deaths). Weight gains good.[5] Reproduction normal.
6	36	At once / In 24 hours Rat pellets 284 / 253 Potatoes 35 / 35 Sweet potatoes 70 / 67 Green beans 64 / 59 Dried peaches 10 / 8 Cheese 279 / 210 Peanuts[3] 85 / 84	Same diet as for Run 5 except pellets and all other foods fumigated for 24 hours with 3 pounds CH_3Br/1,000 cubic feet and fed immediately after fumigation.	Condition excellent (3 deaths). Weight gains good.[5] Reproduction normal. Weight gains equal to controls in Run 5. No significant pathology.

[1] The CH_3Br content (p.p.m. or mg. CH_3Br/kilo of sample) is calculated from the excess Br content of the samples by the method shown in the footnote to Table 1. [2] As rats ate varying quantities of food no estimate can be given of total daily CH_3Br intake. [3] As foodstuffs other than rat pellets were varied from week to week, no estimate can be given of CH_3Br level. At all times either cheese or peanuts were given. This diet simulates a normal human diet, in which are included those materials which absorb the greatest amounts of CH_3Br. [4] See Fig. 1 for weight curves. [5] See Fig. 2 for weight curves.

present in the food, an increased death rate is produced, gain in weight and activity are reduced, and general health and reproductivity are adversely affected. Histopathological examination of the tissues of survivors gave negative pathological findings.

When rat food containing moderate amounts of bromides (30 to 24 mg. Br per 100 gm.), following fumigation at three pounds of methyl bromide per 1,000 cubic feet for 24 hours, or when fumigated fruits and vegetables were fed, little or no deleterious effects were noted. Activity, general condition, gain in weight, and reproductivity were normal. Rat-feeding experiments were carried out in which the diet was made up of a variety of fumigated foodstuffs, i.e., pellets, apples, potatoes, peanuts, cheese, etc. (Fig. 2). No deleterious effects were observed after 20 weeks'

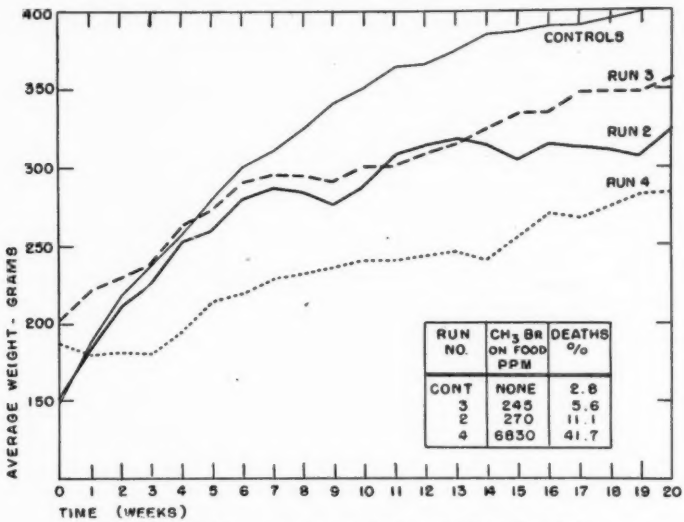

FIG. 1. Weight curves of rats fed on fumigated pellets.

feeding. The diet of these rats approximated more nearly that of humans, and it is noteworthy that cheese and peanuts both were included, both articles absorbing relatively large quantities of methyl bromide.

Rabbit-Feeding Experiments (Table 3 and Fig. 3): Rabbits receiving 56 to 71 mg. of CH_3Br per kilo of body weight in olive oil by intubation showed destruction of superficial layers of stomach, duodenum, and, occasionally, the jejunum, with accompanying hemorrhage and hyperemia. The minimum lethal dose of methyl bromide for rabbits by ingestion is estimated at 60 to 65 mg. of methyl bromide per kilogram of body weight.

When rabbits were fed on a diet containing excessive amounts of organic and inorganic bromides (362 to 256 mg. Br per 100 gm.), all animals died in two weeks, exhibiting symptoms of progressive paralysis. In prolonged rabbit-feeding experiments (52 weeks' duration) the animals were fed on food fumigated at a concentration of three pounds of methyl

bromide per 1,000 cubic feet (9.36 to 5.55 mg. Br per 100 gm.). These rabbits showed little or no deleterious effects. Activity and general condition were normal, but weight increases were 10 to 12 per cent below those shown by the controls.

The lack of appreciable systemic pathological findings in all animals fed foodstuffs fumigated with three pounds of methyl bromide per 1,000 cubic feet is significant, especially since at present in the grain and milling industries a dosage of one pound per 1,000 cubic feet of space is used, Cotton (1941).

The supposition that cooking markedly reduces the quantity of bromide residue, is not substantiated by certain of our findings. In the case

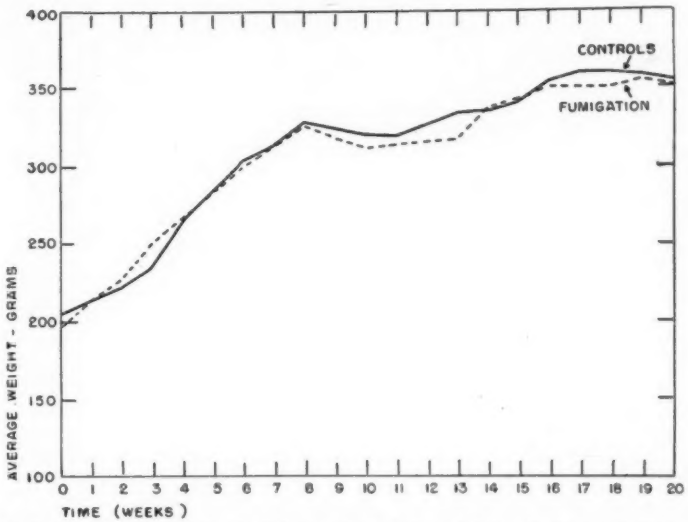

FIG. 2. Weight curves of rats fed on mixed diet containing fruit, vegetables, cheese, and peanuts.

of fumigated flour, we mixed 100-gram portions with water into a stiff dough and baked at 175 to 200°C.(347 to 392°F.) for one hour. The resulting loss of bromide during this procedure approximated 10 per cent of the total amount present. Cooking of dried fruits and of certain fresh vegetables produced little change in the total bromide if the water and juices remained in the sample.

In the experiments reported here, the foodstuffs fumigated for analysis were carried through procedures approximating the usual commercial fumigation practices. Dried fruits, flour, nuts, and cheese were fumigated at two pounds of methyl bromide per 1,000 cubic feet for 24 hours. Fresh fruits and vegetables were fumigated at the same concentrations for two hours. It should, however, be pointed out that, according to Roehm, Shrader, and Stenger (1942) and also Young, Carter, and Soloway (1942), the amounts of bromine retained by a fumigated product depend greatly

TABLE 3

Fifty-Two-Week Rabbit Feedings of Methyl Bromide Fumigated Materials

Run No.	Number of rabbits on test	CH_3Br content of food (p.p.m.)[1]	Treatment of food and feeding program[2]	Results
2	12	None (unfumigated)	100 grams pellet-oats mixture per animal per day. 100 grams alfalfa hay per animal 3 times per week. Unfumigated.	Three deaths. General condition excellent. Normal weight increases. All deaths due to ear infection.
1	12	(Time fed after fumigation) Pellets—oats: At once, 3,720 In 24 hours, 3,060 Alfalfa hay: At once, 4,350 In 24 hours, 4,330 Average, 3,865	100 grams pellet-oats mixture per day. 100 grams alfalfa hay 3 times per week. All food fumigated with undiluted CH_3Br gas, at atmospheric pressure, for 24 hours. Fed immediately after fumigation.	All dead in 2 weeks. Death preceded by marked decrease in food intake and marked drop in weight. In 3 to 5 days before death, progressive paralysis was noted in all animals, beginning in hind quarters and progressing forward. Death occurred without convulsions. Pathologic examination showed cerebral changes; secondary pulmonary damage in all.
3	12	Pellets—oats: At once, 100 In 24 hours, 97 Alfalfa hay: At once, 86 In 24 hours, 81 Average, 90	100 grams pellet-oats mixture per day. 100 grams alfalfa hay 3 times per week. All feed fumigated 24 hours at concentration of 3 lbs. CH_3Br/1,000 cu. ft. Food fed immediately after fumigation.	No deaths. General condition excellent. Average weight increases good but 10 per cent less than controls. Marked increase in water intake and urine excretion. Estimated average intake of CH_3Br per animal per day = 13.5 mg. CH_3Br. No significant histopathology.[3]
4	12	Pellets—oats: In 24 hours, 97 In 48 hours, 82 Alfalfa hay: In 24 hours, 81 Average, 80	Same feeding schedule as in Run 3 but food and hay aerated 24 hours after fumigation, and then fed.	Three deaths. General condition excellent. Average weight increases good but 11 per cent less than controls. Marked increase in water intake and urine excretion. Estimated average intake of CH_3Br per animal per day = 12 mg. CH_3Br. No significant histopathology.[3] 2 deaths due to ear infection; 1 death due to intestinal obstruction.

[1] The CH_3Br content (p.p.m. or mg. CH_3Br/kilo of sample) is calculated from the excess Br content of the samples by the method shown in the footnote to Table 1. [2] Pellets were a balanced, commercially prepared rabbit food. Pellets and whole-grain oats were mixed half and half, and 100 grams of the mixture fed to each animal per day. This amount was regularly completely consumed except in the case of the animals dying in two weeks. Dried alfalfa hay was fed three times per week, 100 grams per animal per day. [3] The estimated daily apparent intake of CH_3Br per animal is calculated from the excess Br on the food by striking a general average. This is based on the assumption that all excess Br is present as CH_3Br. The values provide a basis of comparison for the possible intake of man when eating such foods as are shown in Table 1; see Fig. 3 for weight curves.

upon the nature of the material and upon the conditions under which fumigation takes place, in particular, upon the concentration of methyl bromide, ratio of methyl bromide to product, time of exposure, temperature, type of fumigation (vacuum, atmospheric), and relative humidity in the case of the atmospheric type. It is difficult, therefore, to duplicate commercial conditions in the laboratory.

The fumigation of materials used in the animal-feeding experiments was, in all cases, at a concentration (three pounds of methyl bromide per 1,000 cubic feet) above those usually used in actual practice. Likewise, the period of fumigation in all cases was 24 hours. These facts explain

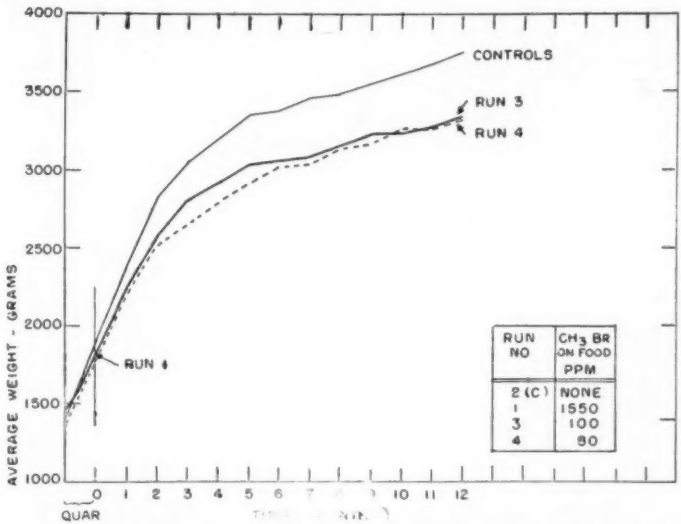

FIG. 3. Weight curves of rabbits fed on fumigated hay and pellets.

why the bromide content of the rat feed was higher than that of corresponding foodstuffs fumigated by the usual procedure.

In actual practice the use of excessive concentrations of methyl bromide or of lengthy fumigation periods is discouraged because fresh fruits and vegetables are harmed by such procedures. In the case of other foodstuffs, the tendency is to use the lowest effective concentration because of the cost of the fumigant. Excessive and rapid deterioration of the fresh produce is caused by high dosage, prolonged fumigation, or high temperatures. These conditions also tend to cause excessive absorption of the fumigant.

From the results of Roehm, Shrader, and Stenger (1942), Cotton (1941), and Young, Carter, and Soloway (1942) it has been demonstrated that commercial fumigation of flour, cheese, and nut meats is carried out at concentrations of one pound CH_3Br per 1,000 cubic feet which is one-half the concentration used in our experimental procedures (Table 1). Thus it would seem that commercial practices are tending to reduce

the concentration of fumigant, which is economy in the right direction since it also reduces the amount of bromide residue remaining on the fumigated products.

The diets fed to the animals in the tests herein described consisted entirely of fumigated foodstuffs, all of which contained more organic and inorganic bromides than are found in similar foodstuffs fumigated by methods approximating commercial procedures. Thus it seems unlikely that the small amount of methyl bromide or bromide residues on commercially fumigated fresh vegetables and fruits, or dried fruits, is harmful to the consumer. For the past three years ever-increasing amounts of these products have been commercially fumigated, and no report has been made of any untoward effects.

Laug (1941) and Roehm, Shrader, and Stenger (1942) have shown that the bromide retained by cereal products following fumigation with methyl bromide exists almost entirely in inorganic form or nonvolatile bromine after standing for a few days. As pointed out above, the amounts retained are likely to be smaller in commercial fumigation than in laboratory fumigation. Hanzlik, Talbot, and Gibson (1928) showed that feeding young rats a modified Osborne-Mendel diet containing .0054 per cent sodium bromide (5.4 mg. per 100 gm.) over a period of seven months caused no toxic signs or symptoms except a moderate loss of body weight (11 per cent) and of food consumption (2.2 per cent).

Clark, Adams, and Morrissey (1940) fed dogs twice daily 22.5 mg. per kilo body weight of sodium bromide over a period of three months without noting any marked blood changes or alterations of the electrocardiogram. Similarly, Calvery (personal communication) saw no untoward effects in dogs (only two dogs at each level were studied) following the oral administration of 20 mg. and 200 mg. of sodium bromide per kilo daily over a period of 18 weeks. However, the animals were not sacrificed. Our animal experiments on feeding rats food containing 24 to 30 mg. of bromine per 100 gm. of food for 20 weeks caused little or no deleterious effects. Similarly, feeding experiments with rabbits for a period of 52 weeks with food containing from 5.55 to 9.36 mg. of bromine per 100 gm. of food caused little or no deleterious effect. These foods were fumigated with three pounds of CH_3Br per 1,000 cubic feet under laboratory conditions and had, therefore, a higher bromine content than found under commercial conditions. It appears, therefore, that such amounts of bromine as encountered in foods fumigated with methyl bromide, under commercial conditions, would, on the basis of our evidence, probably not contain sufficient quantities of bromine residues to produce deleterious effects. For this reason milled cereal products fumigated with one pound of methyl bromide per 1,000 cubic feet will probably offer no risk when such food is consumed.

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A FUNGISTATIC MEDIUM FOR ENUMERATION OF YEASTS[1]

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In a number of food industries the control of yeast contamination is of utmost importance. This is particularly true in the nonalcoholic beverage industry where a large proportion of such biological deterioration as may occur is associated with yeast growth. Molds, on the other hand, though of considerable significance as possible agents of deterioration in still beverages, are of minor significance in carbonated beverages. Bacteria are of particular significance in the nonacid or low-acid products.

A systematic study of the sources of these various types of microorganisms soon impresses one with the fact that though it is readily feasible to estimate the numbers of bacteria as compared with yeasts and molds, it has been extremely difficult to ascertain the numbers of yeasts and molds independently because the media generally employed for the detection of one are about equally satisfactory for the growth of the other. In mixtures of yeasts and molds, when employing such standard media as acid dextrose agar and malt extract or wort agar, the molds generally grow so much more vigorously than the yeasts that they tend to overgrow the plate with the resultant extreme difficulty in estimating the number, if not completely preventing the growth, of yeasts. As yeast contamination is of primary significance in the beverage industry and other food industries, it became evident that if any dependable estimation of yeasts and molds was to be made in such basic substances as sugar and color solutions or such environmental and mechanical structures as washing machines, brushes, plant air, etc., that the development of a medium which would permit the growth of yeasts and inhibit, if not completely prevent, growth of molds, for at least three or four days, was essential. Such a medium would be of value not only in the beverage industry but in every food industry and for the examination of every product in which a differential count of yeasts and molds is desired, or for the purpose of isolation of yeast, and possibly bacteria, from mold contamination.

A search of the literature did not disclose any studies toward the development of such a medium, and inquiry to several biological supply houses elicited the information that they were not aware of the existence of any such selective media. There were many references in the literature, however, to studies on substances which inhibit mold growth and a few of these are reviewed below.

Moran, Smith, and Tompkins (1932) studied the effect of carbon dioxide as a preservative for meat. They found that yeasts and bacteria grew much more readily than did molds in an atmosphere containing 20 per

[1] This study was made possible through a fellowship maintained at the Iowa State College by the American Bottlers of Carbonated Beverages.

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cent carbon dioxide and that a 10-per cent concentration of carbon dioxide markedly reduced the intensity of growth of many molds.

Kiesel (1913), in testing the effect of various organic acids and their salts on the germination, growth, and formation of conidia of *Aspergillus niger*, found that monobasic acids were more effective than dibasic and tribasic acids and that the inhibitory effect of monobasic acids increased with increasing molecular weight. Monochloracetic acid had about the same effect as propionic acid.

Kirby, Atkin, and Frey (1937) reported that acetic acid inhibited the growth of *Aspergillus niger* and *Rhizopus nigrificans* to a greater extent than did calcium acid phosphate, citric acid, lactic acid, and tartaric acid.

Hoffman, Schweitzer, and Dalby (1939) reported that the inhibition of mold growth by an organic acid increased with the length of the carbon chain, with the concentration of the acid in the medium, and with increasing hydrogen-ion concentration.

Macy and Olson (1939) reported the successful use of wrappers of parchment paper soaked in solutions of calcium or sodium propionate for the inhibition of mold growth on the surface of butter.

Tompkins, in a series of reports of the Food Investigation Board of Great Britain's Department of Scientific and Industrial Research (1935, 1936, 1937), compared the relative fungistatic properties of a large number of organic compounds when used for impregnating wrappers for the inhibition of the growth of molds on oranges. Of the many compounds tested, diphenyl appeared to be most promising. A number of the compounds were found to have fungistatic properties but, because of their physical properties and tendencies toward the production of off-odors and tastes, they were not considered applicable as food preservatives. However, it was thought that some of these compounds might be effective in inhibiting mold growth when added directly to a culture medium.

A method developed by Farkas (1939), employing diphenyl for impregnating wrappers, was found to markedly reduce mold deterioration of oranges shipped from Palestine.

EXPERIMENTAL PROCEDURE

After some preliminary observations it was decided that sodium propionate and diphenyl would be worthy of investigation to ascertain whether they had a selective fungistatic action against molds as compared with their inhibitory properties with regard to the growth of yeasts.

The basal medium employed consisted of the following:

Malt extract (Difco)	20 grams
Peptone (Difco)	1 gram
Ammonium chloride	1 gram
K_2HPO_4	1 gram
Agar	20 grams
Water	1,000 ml.
Lactic acid to bring the reaction to pH 4.5-4.7. (7.0 ml. of N/1 lactic acid solution for control medium)	

The basal ingredients were dissolved in the required quantities of water and the medium dispensed in 100-ml. portions in bottles, the reaction was adjusted to pH 4.5-4.7 with lactic acid, then the bottles were sterilized (15

minutes at 12 pounds of pressure), and plates were poured immediately after removal from the autoclave. This constituted the control medium. Fungistatic compounds were added in the desired concentrations after the liquefied medium had been cooled to a temperature of 50 to 60°C.(122 to 140°F.), and plates were immediately poured.

Sixteen known cultures of molds, furnished by Professor J. C. Gilman of the Department of Botany at Iowa State College, and 29 species of yeasts, which were supplied by Dr. E. M. Mrak of the Division of Fruit Products, University of California, Berkeley, California; together with several unidentified molds isolated from air and about 75 yeasts from carbonated beverages were employed as test organisms.

OBSERVATIONS WITH SODIUM PROPIONATE

Propionic acid was neutralized with sodium hydroxide to pH 5.8 as determined by measurements with a Coleman model 3D pH electrometer, pH 5.8 having been found to be the point of inflection on the titration curve of propionic acid. The desired quantities of this propionic acid solution were then added to the malt extract agar basal medium which was then acidified with lactic acid to bring the reaction to pH 4.7 ± .5; the medium was then sterilized in the autoclave at 12 pounds of pressure for 15 minutes and immediately poured into petri dishes. Concentrations of propionic acid employed were 1,000, 2,000, and 3,000 p.p.m. (.1, .2, and .3 per cent).

The media in the petri dishes prepared as above were inoculated with the various test yeasts in the following manner. The yeasts were transferred from stock malt extract agar stabs into malt extract broth, and after 48 hours' incubation in this medium at 28°C.(82.4°F.), a loopful of the culture was streaked onto the surface of the test agar medium. Molds were inoculated by touching the surface of the test medium with a needle impregnated with spores of the test mold. Inoculation of molds was made at four points on the plate, rather than on a single point, in order to reduce the chance of failure of inoculation. Care was taken to prevent as far as possible, transfer of vegetative filaments and inadvertent shaking of mold spores onto the medium at points other than those it was desired to touch with the inoculating needle.

The inoculated solid media in the petri dishes were then incubated at 28°C. and vigor of growth was reported after two, three, and four days. The records were as follows:

For yeasts

—	no growth visible macroscopically;
±	faint signs of growth;
+	isolated colonies less than .5 mm. in diameter;
++	isolated colonies .5 to 1.0 mm. in diameter;
+++	isolated colonies 1.0 to 2.0 mm. in diameter.

For molds

—	no visible growth;
±	faint signs of growth;
+	isolated colonies less than three mm. in diameter;
++	isolated colonies three to five mm. in diameter;
+++	isolated colonies five to 10 mm. in diameter;
++++	isolated colonies greater than 10 mm. in diameter.

Although observations were made for two, three, and four days, only the four-day results are included for consideration and discussion (Tables 1 and 2). The shorter periods of incubation merely showed less vigorous growth of yeasts and molds.

It is apparent that all of the molds were inhibited by .3 per cent (3,000 p.p.m.) sodium proportionate whereas a number of the yeasts grew. However this concentration was evidently too great to employ as a differential agent, since many of the yeasts failed to grow after a period of four days or were markedly inhibited.

TABLE 1

Effect of Sodium Propionate on Growth of Molds on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.), Four Days

Concentration of sodium propionate....		None	1,000 p.p.m.	2,000 p.p.m.	3,000 p.p.m.
Lab. No.	Designation	Vigor of growth			
11	*Aspergillus niger*	++++	++++	++++	—
12	*Aspergillus terreus*	++++	+++	++	—
13	*Aspergillus fumigatus*	++++	++++	++++	—
14	*Aspergillus ochraceus*	++++	++++	±	—
15	*Penicillium expansum*	+++	++	±	—
16	*Penicillium exalicum*	++++	+	±	—
17	*Penicillium echinatum*	++++	+++	±	—
18	*Penicillium humicola*	++++	+++	++	—
19	*Rhizopus nodosus*	++++	++++	++++	—
20	*Rhizopus nigrificans*	++++	++++	+++	—
21	*Mucor hiemalis*	++++	+	±	—
22	*Mucor racemosus*	++++	++++	+	—
23	Cunninghamella species	++++	+++	—	—
24	Hormodendron species	++++	+	—	—
25	*Fusarium niveum*	++++	++	—	—
26	*Fusarium graninearum*	++++	—	—	—

The observations on the fungistatic properties of various concentrations of sodium propionate with respect to the degree of inhibition of 16 cultures of various species of molds and 26 yeast strains are summarized (Table 3). With 2,000 p.p.m. sodium propionate, 75 per cent of the molds were moderately or very markedly inhibited, but 43 per cent of the 26 yeasts were similarly affected. Although this concentration showed some selective inhibition against molds as compared with yeasts, the differences in inhibiting properties were not as clear-cut as necessary to warrant its use.

With the higher concentration (3,000 p.p.m.) all of the molds were prevented from growing in four days but 11, or 42 per cent, of the yeasts were also markedly or completely inhibited. On the other hand, going to a lower concentration (1,000 p.p.m.) which was not inhibitory to yeasts (only seven per cent moderately inhibited), the retarding effect on growth of molds was not sufficient to warrant its use as a selective inhibitory medium since 63 per cent of the molds were not inhibited to any extent.

It is perhaps of interest and significance to note that the molds *Aspergillus niger, Aspergillus fumigatus, Rhizopus nodosus,* and *Rhizopus nigrificans* were very resistant to sodium propionate as compared with the other molds, since they grew quite luxuriantly (about as vigorously as in

the controls) in the presence of 2,000 p.p.m. sodium propionate. All of the other molds were very markedly inhibited or prevented from growing by this concentration. It appears, therefore, that although sodium propionate was effective against many more molds than yeasts, the concentration necessary to appreciably inhibit all of the molds was such as to rather markedly inhibit many of the yeasts that are likely to be encountered so that this compound could not be satisfactorily employed as a selective fungistat.

TABLE 2

Effect of Sodium Propionate on Growth of Yeasts on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.), Four Days

Sodium propionate concentration		None	1,000 p.p.m.	2,000 p.p.m.	3,000 p.p.m.
Lab. No.	Designation	Vigor of growth			
201	*Zygosaccharomyces prioranus*	++++	++++	++	+
202	*Zygosaccharomyces pastori*	+++	++	±	—
203	*Zygosaccharomyces Barkeri*	++	+	±	—
204	*Kloeckera austriaca*	+++	+++	+	—
205	*Hansenula anomala*	+++	+++	+++	+++
206	*Mycoderma decolerens*	++	++	++	+
207	*Mycoderma vini*	++	+	+	+
208	*Rhodotorula rubra*	+++	+++	+	—
209	*Debaromyces Guilliermondii*	+++	+++	+	—
210	*Debaromyces membranaefaciens*	+	—	—	—
211	*Hanseniaspora Melligeri*	++++	++++	++++	++
213	*Saccharomyces ellipsoideus* (Hansen)	++++	++++	++++	+++
214	*Saccharomyces ellipsoideus* (Hansen, Rasse, Champagne)	++++	+++	++	+
215	*Saccharomyces ellipsoideus* (Hansen, Rasse, Johannisberg I)	++	++	+	±
216	*Saccharomyces ellipsoideus* (Hansen, Rasse, Johannisberg II)	++++	+++	++	+
217	Saccharomyces ellipsoideus	++++	++++	++++	++++
218	Saccharomyces ellipsoideus	++++	+++	+++	++
219	*Saccharomyces cerevisiae* var. *ellipsoideus*	++++	++++	++++	++++
220	*Torulopsis monosa*	++++	++++	++++	++++
222	*Torulopsis dattila*	+++	++	++	++
223	*Saccharomyces italicus*	++++	++++	++++	++++
224	*Saccharomyces fragilis*	++++	++++	++++	++++
225	*Zygopichia californica*	++++	++++	++++	++
226	*Schizoblastosproion Starkeyi-henrici*	+++	++	++	++
227	*Saccharomycodes ludwigii*	++++	++++	+++	+++
229	*Schwanniomyces occidentalis*	++++	++	++	—

OBSERVATIONS WITH DIPHENYL

Diphenyl is a colorless, crystalline, organic compound of the formula $C_6H_5C_6H_5$ consisting, as the name indicates, of two phenyl groups joined together. It is volatile and has a very distinctive odor resembling that of naphthaline. Eastman practical-grade diphenyl was used for all tests described below. As the compound is only slightly soluble in water, alcoholic solutions (95 per cent ethyl alcohol) were employed.

The basal medium, the test organisms, and technics of inoculation in the studies with diphenyl were the same as that previously described for

sodium propionate except that, the diphenyl being volatile and only very slightly soluble in water, alcoholic solutions of the compound were employed. After the sterile malt extract agar, distributed in 100-ml. portions in bottles, had been cooled to 40 to 45°C.(104 to 113°F.), the desired volume of the alcoholic solution of diphenyl was added by means of a one-ml. pipette graduated in .1 ml.; the mixture was thoroughly agitated until the diphenyl (which crystallized in very fine particles as soon as it came in contact with the medium)) was evenly and thoroughly dispersed throughout the agar which was then quickly poured into petri plates and cooled.

TABLE 3

Relative Inhibitory Properties of Sodium Proprionate on Molds and Yeasts on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.), Four Days

Degree of growth inhibition.....	None		Slight		Moderate		Marked or complete	
Concentration of sodium propionate	No.	(%)[1]	No.	(%)[1]	No.	(%)[1]	No.	(%)[1]
MOLDS								
1,000 p.p.m.	6	(38)	4	(25)	2	(12)	4	(25)
2,000 p.p.m.	3	(19)	1	(6)	2	(12)	10	(63)
3,000 p.p.m.	....	....	....	....	....	....	16	(100)
YEASTS								
1,000 p.p.m.	16	(62)	8	(31)	2	(7)	....	....
2,000 p.p.m.	10	(38)	5	(19)	10	(39)	1	(4)
3,000 p.p.m.	5	(19)	7	(27)	3	(12)	11	(42)

[1](%) To nearest whole number.

After a series of preliminary observations employing a few yeasts and molds it was concluded that 100 p.p.m. (.01 per cent)was the most desirable concentration to employ. All of the molds observed in the preliminary studies, with the exception of *Rhizopus nigrificans* and an unidentified species from the air, were markedly inhibited by 100 p.p.m., whereas 50 p.p.m. permitted luxuriant growth of all the molds in four days. On the other hand, 100 p.p.m. of diphenyl did not appreciably decrease the size of colonies of any of the species of yeasts employed in the preliminary observations if incubation was for three to four days. The members of the genera Saccharomyces, Torulopsis, and Saccharomycodes grew luxuriantly in a concentration of 150 p.p.m. diphenyl, but *Zygosaccharomyces Barkeri* and *Debaromyces guilliermondii* were inhibited by this concentration. It appeared, therefore, that 100 p.p.m. might be a suitable concentration for effective fungistatic action against molds without appreciably interfering with growth of yeasts.

The relative vigor of growth after two and four days on the control medium without diphenyl and in the presence of 100 p.p.m. diphenyl is shown (Table 4) for 16 known species of molds and five unidentified molds obtained from various sources; and the results for 27 species of yeasts are given (Table 5).

It will be noted (Table 4) that two species of Rhizopus and one unidentified mold from the air were not appreciably inhibited by 100 p.p.m.

diphenyl. Other experiments indicated that even 500 p.p.m. did not markedly inhibit these molds.

Though a number of yeasts were inhibited during the first two days of growth (Table 5), very few showed any appreciable inhibition of growth after four days. In addition to the identified yeasts there are available 75 strains of yeasts isolated from carbonated beverages. Observations on the inhibitory properties of 100 p.p.m. diphenyl on the 21 cultures

TABLE 4

Effect of Diphenyl on Growth of Molds on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.)

Incubation period		2 days		4 days	
Concentration of diphenyl		None	100 p.p.m.	None	100 p.p.m.
Test mold		Vigor of growth			
Lab. No.	Designation				
1	From air	+++	+	++++	+++
6	Apparently Penicillium	++++	—	++++	±
7	Unknown pink mold	++++	—	++++	—
8	Apparently Mucor	++++	—	++++	+
10	Apparently Mucor	++++	—	++++	±
11	*Aspergillus niger*	++++	±	++++	++
12	*Aspergillus terreus*	+++	±	++++	+
13	*Aspergillus fumigatus*	++++	—	++++	++
14	*Aspergillus ochraceus*	++	±	++++	+
15	*Penicillium expansum*	+	—	++	—
16	*Penicillium oxalicum*	+	±	+	±
17	*Penicillium schinatum*	+++	±	++++	+
18	*Penicillium humicola*	++	±	+++	±
19	*Rhizopus nodosus*	++++	+++	++++	++++
20	*Rhizopus nigrificans*	++++	+++	++++	++++
21	*Mucor hiemalis*	++++	—	++++	+
22	*Mucor racemosus*	++++	±	++++	+
23	Cunninghamella species	++++	±	++++	++
24	Hormodendron species	—	—	++	±
25	*Fusarium niveum*	+++	±	++++	++
26	*Fusarium graminearum*	+++	—	++++	+

of molds, 27 identified yeast strains, and 75 strains isolated from carbonated beverages are summarized (Table 6).

The selective fungistatic action of 100 p.p.m. diphenyl is rather strikingly indicated in the data shown (Table 6). Whereas among 20 molds observed for a two-day period 90 per cent were inhibited, and of 21 molds observed for a four-day period 86 per cent fell into this category, the reverse phenomenon was observed with the yeasts. Thus, of 100 yeast strains which grew well on the control medium in two days 17 per cent were markedly inhibited; whereas, of 102 yeasts which grew well in four days on the control medium only four per cent were moderately or appreciably inhibited in the presence of 100 p.p.m. diphenyl. As has previously been indicated, the molds which were not inhibited belonged to the genus Rhizopus.

There were several instances in which the vigor of growth of the yeasts was found to be greater in the presence of 100 p.p.m. diphenyl than on

TABLE 5

Effect of Diphenyl on Growth of Yeasts on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.)

Incubation period		2 days		4 days	
Concentration of diphenyl		None	100 p.p.m.	None	100 p.p.m.
Test yeast		Vigor of growth			
Lab. No.	Designation				
201	*Zygosaccharomyces prioranus*	+++	++	++++	++++
202	*Zygosaccharomyces pastori*	++++	+++	++++	+++
203	*Zygosaccharomyces Barkeri*	+++	+++	++++	+++
204	*Kloeckera austriaca*	++++	+++	++++	++++
205	*Hansenula anomala*	++++	+++	++++	++++
206	*Mycoderma decolorens*	++++	++++	++++	++++
207	*Mycoderma vini*	++	++	+++	+++
208	*Rhodotorula rubra*	++++	+++	++++	+++
209	*Debaromyces Guilliermondii*	++++	+++	++++	++++
210	*Debaromyces membranaefaciens*	+++	—	+++	±
211	*Hanseniaspora Melligeri*	++++	+++	++++	++++
213	*Saccharomyces ellipsoideus* (Hansen)	++++	++++	++++	++++
214	*Saccharomyces ellipsoideus* (Hansen, Rasse, Champagne)	++++	+++	++++	++++
215	*Saccharomyces ellipsoideus* (Hansen, Rasse, Johannisberg I)	++++	+++	++++	++++
216	*Saccharomyces ellipsoideus* (Hansen, Rasse, Johannisberg II)	++++	++	++++	+++
217	*Saccharomyces ellipsoideus*	++++	++++	++++	++++
218	*Saccharomyces ellipsoideus*	++++	++++	++++	++++
219	*Saccharomyces cerevisiae* var. *ellipsoideus*	++++	++++	++++	++++
220	*Torulopsis monosa*	++++	++	++++	+++
221	*Schizosaccharomyces octosporus*	—	—	+++	+++
222	*Torulopsis dattila*	++++	++++	++++	++++
223	*Saccharomyces italicus*	++++	++++	++++	++++
224	*Saccharomyces fragilis*	++++	++++	++++	++++
225	*Zygopichia californica*	++++	+++	++++	+++
226	*Schizoblastosporion Starkeyi-herrici*	+++	+++	+++	+++
227	*Saccharomycodes ludwigii*	++++	++++	++++	++++
229	*Schwanniomyces occidentalis*	++++	++	++++	+++

TABLE 6

Relative Inhibitory Effects of Diphenyl on Molds and Yeasts on Malt Extract Agar—pH 4.7, 28°C.(82.4°F.)

Incubation period	2 days[1]	4 days	2 days	4 days
Degree of inhibition by 100 p.p.m. diphenyl	Number of molds		Per cent of molds	
None	....	2	....	9
Slight	2	1	10	5
Moderate	4	5	20	24
Marked to complete	14	13	70	62
	Number of yeasts		Per cent of yeasts	
None	40	73	40	72
Slight	33	25	33	24
Moderate	13	3	13	3
Marked to complete	4	1	4	1

[1] One mold and two yeasts did not grow on control medium in two days but grew in four days.

the control plates. It is of interest to note that some of the yeasts, culture No. 208, *Rhodotorula rubra* and a pink yeast isolated from sugar, produced a very intense pigment in the control medium but in the presence of 100 p.p.m. diphenyl no color was formed. Sodium propionate did not affect color production by these yeasts. On the other hand, whereas *Aspergillus niger* produced characteristic black conidia on the standard control medium, conidia formation in the presence of 100 p.p.m. diphenyl was very markedly delayed and the vegetative hyphae were distinctly yellow. Spores were produced only after the diphenyl had evaporated and at that time the yellow color of the vegetative hyphae had disappeared or was masked. This influence of diphenyl on pigment formation might be of interest in subsequent studies on physiological characteristics of yeasts and molds.

The fungistatic action of 100 p.p.m. diphenyl and its relative lack of influence against the growth of yeasts are illustrated (Figs. 1 to 5). The period of incubation of the plates from which these photographs were made was two and one-half days (60 hours) at 28°C.(82.4°F.).

SUMMARY

A malt extract agar medium into which was incorporated 100 p.p.m. diphenyl was found to exert marked fungistatic action on a large variety of molds for a period of 72 to 96 hours, whereas, with very few exceptions, yeasts grew luxuriantly. Several strains of Rhizopus were found to be only slightly inhibited by diphenyl in concentrations up to 500 p.p.m.

Sodium propionate, in a concentration of 3,000 p.p.m., inhibited all of the molds that were tested but many of the yeasts were also markedly inhibited. Lower concentrations (2,000 to 1,000 p.p.m.) of sodium propionate were less detrimental to the yeasts but they were not sufficiently inhibitory against the molds to warrant its use as a selective inhibitory agent for the isolation or enumeration of yeasts in mixtures with molds.

Diphenyl (100 p.p.m.) has been satisfactorily employed for the enumeration of yeasts and suppression of molds in air analysis and is now being studied with a view to enumeration of yeasts in such products as sugars and other constituents of food products which may harbor molds.

Diphenyl (100 p.p.m.) in nutrient and malt extract agar has been employed successfully in this laboratory to recover bacterial and yeast cultures which had become contaminated with molds.

In the presence of diphenyl a pink yeast failed to produce pigment; spore formation by *Aspergillus niger* was suppressed and its vegetative mycelium was intensely yellow.

The insolubility of diphenyl renders its use inconvenient and its volatility results in loss of fungistatic properties on prolonged incubation. Studies are now in progress with a view to discovering some nonvolatile, water-soluble substance that possesses selective, fungistatic properties similar, if not superior, to diphenyl.

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With 100 p.p.m. diphenyl Without diphenyl With 100 p.p.m. diphenyl Without diphenyl

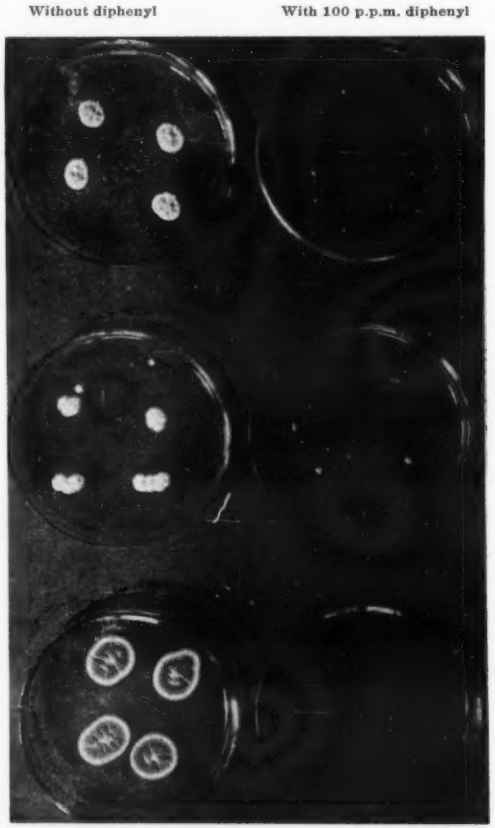

FIG. 1. Growth of molds on malt extract agar.

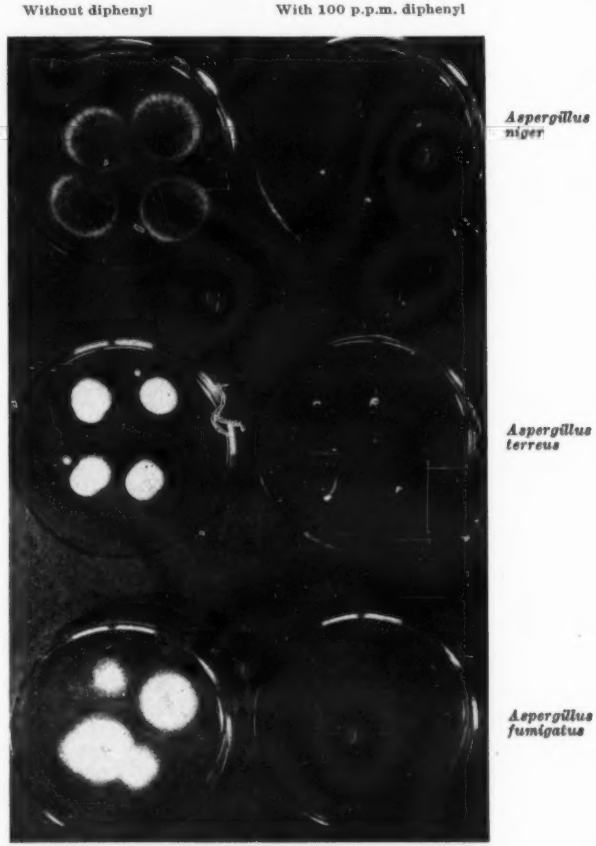

FIG. 2. Growth of molds on malt extract agar.

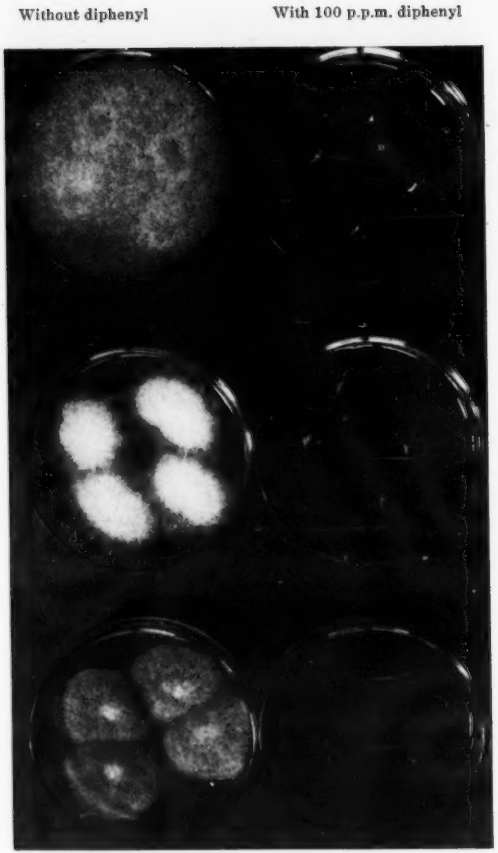

FIG. 3. Growth of molds on malt extract agar.

FIG. 4. Growth of molds on malt extract agar.

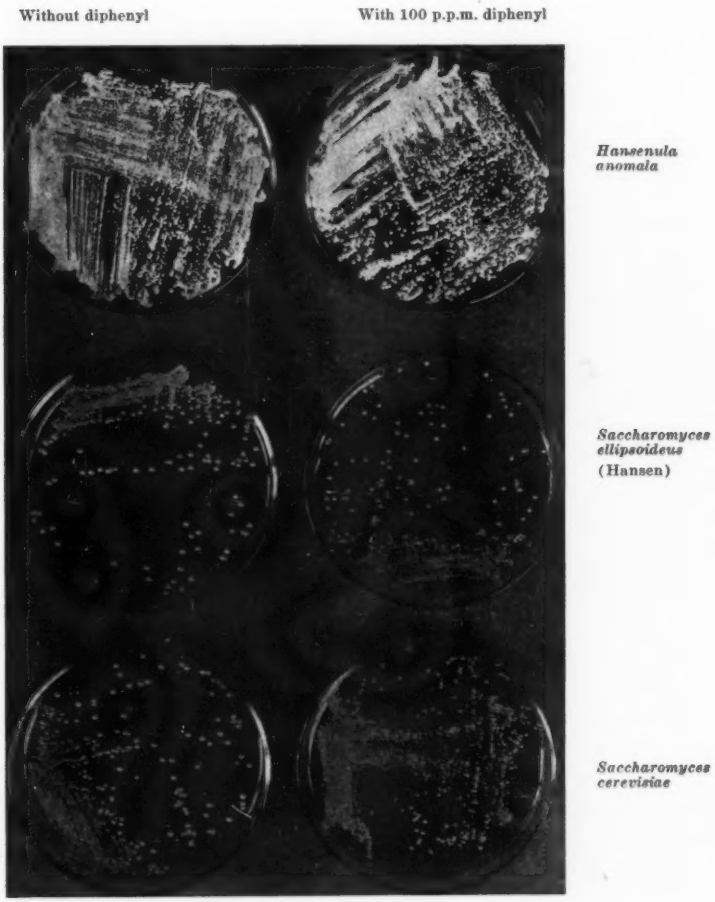

FIG. 5. Growth of yeasts on malt extract agar.

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FIG. 4. Growth of molds on malt extract agar.

FIG. 3. Growth of molds on malt extract agar.

A MICROBIOLOGICAL ANALYSIS OF APPLE JUICE PROCESSING

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The production and preservation of apple juice is rapidly becoming one of America's important food industries. Mottern, Nold, and Willaman (1941) reported a pack of 4,400,000 gallons definitely accounted for in 1940 and a probable total output of at least five to six million gallons in the United States. The American Fruit Grower magazine (1941) cited the production of apple juice in Canada as 3,000,000 gallons in 1940, compared with 400,000 gallons in the previous year. It is reasonable to expect further increases. Since apple juice processing is new in the canning industry, little work has been published relative to its bacteriology. It is with this in mind that this study of the bacteriology involved in the production and preservation of apple juice is presented.

The problems specifically studied were (1) the effect of washing the apples upon the microbiological count; (2) the microbiological count of certain pieces of equipment; (3) the effect of certain procedures and equipment upon the microbiological count of the juice during processing; and (4) the effect of freezing upon the microflora. These problems are described more in detail in the following sections. Processes of manufacture were adopted or devised, and bacteriological studies were carried out at appropriate points.

REVIEW OF LITERATURE

Tanner (1932) reported a ropiness in cider owing to the growth of certain organisms which generally are unable to grow in the presence of acid; he found that ropiness develops before the cider becomes too acid for the further development of the organisms. In cans and jars which he obtained from several canneries Fellers (1928) reported the presence of cocci, aerobic sporeforming bacilli, molds, yeasts, and some undetermined organisms. Carpenter, Pederson, and Walsh (1932), in a study of sterilization of apple juice by filtration, stated that Seitz germ-proof filtering is possible under proper operating conditions. In their study on the preservation of cider, Fabian and Marshall (1935) found that the pasteurizing time of cider was determined to a certain extent by the number and type of organisms present in the juice. The vegetative forms of yeast were killed at a temperature of 65.5°C.(150°F.) in 10 minutes. The most resistant mold spores were killed by pasteurization at a temperature of 79°C.(175°F.) for 20 minutes and certain bacteria at 70°C.(158°F.) for 30 minutes or 80°C.(176°F.) for 15 minutes. Acidity was also mentioned as a very important factor in determining the time and temperature to be used in pasteurization. Tressler and Pederson (1938) advocate complete filling of containers since flash heating does not destroy all mold spores, which cause spoilage as long as free oxygen is present. Tempera-

tures even as high as 82.2 to 85°C.(180 to 185°F.) for one minute did not destroy mold spores. As a result of their work previous to 1938 Marshall and Kremer (1937) recommend the addition of hot juice at 87.8°C. (190°F.) to clean, warm, but unsterilized bottles or cans as a safe method of preservation. Concerning holding-pasteurization Joslyn and Marsh (1937) state that, although apple juice pasteurized at 60°C.(140°F.) for 30 minutes was rid of organisms and was of better flavor than juice heated at higher temperatures, this treatment did not destroy pectic enzymes. Pederson and Tressler (1938), in a thorough study of flash pasteurization of apple juice, make the following recommendations: (1) apple juice prepared from tart or acid eastern apples can be flash pasteurized and canned at a temperature of 71°C.(160°F.) or higher; (2) it is not necessary to destroy all organisms in juice, as the absence of air prevents mold development; (3) malic acid added to the apple juice aids in killing bacteria; and (4) the acidity of the juice, in addition to other factors, plays an important role in determining the time and temperature of pasteurization.

DESCRIPTION OF PROCESS

For the sake of clarity the diagram (Fig. 1) is used as a reference for the description of the process followed in the bacteriological experiments to be described.

In brief, the apples were macerated by means of an apple grater. The macerated apples were collected in cloths placed between wooden racks and pressed by means of a hydraulic press. The juice, which was collected in a large wooden tank, was strained through a cheesecloth to remove the larger particles and was either rough filtered by passing it through a special type of filter disc in a power filter, by which method a cloudy product was obtained, or treated in one of two other ways, described as follows:

One alternate method of treating the unfiltered juice involved the introduction of a special form of diatomaceous earth (Hyflo Super-Cel or Dicalite Speedex) into the juice. After the mixture was thoroughly agitated it was passed through a power filter. The juice emerged as a clear polished product, while the solid material from the juice plus the diatomaceous earth was left on the filter discs.

The other method differed from the one just described in that the unfiltered juice was treated with a pectin-hydrolyzing enzyme, Pectinol A, and allowed to react at a cool temperature, approximately 3.3°C. (38°F.), for about 22 hours. (The cool temperature was preferred so as to reduce fermentation from microorganisms to a minimum.) After being allowed to react, the juice was either rough filtered to a cloudy juice or mixed with diatomaceous earth and treated as described above (Fig. 1). The advantage of using Pectinol A was that it made the juice easier to filter. Furthermore, apple juice low in pectin content, either naturally or by means of a pectin-splitting enzyme, according to Joslyn and Marsh (1937), may be pasteurized at a lower temperature than juice which is high in pectin.

In case the juice could not be processed the same day it was pressed, it was placed in clean jugs or other vessels and allowed to stand at low

cold-storage temperatures—2.2 to 3.3°C. (26 to 38°F.)—or frozen solid. The frozen juice could be treated by one of the methods described above; that is, it could be either rough filtered or clarified by using Pectinol A in conjunction with the filter air, or by using diatomaceous earth alone. (The author found that juice which had once been frozen and thawed filtered much more easily than unfrozen juice. Possibly the precipitation of colloidal material by freezing accounted for the ease of filtration.)

After the juice was filtered or clarified, it was pumped through a flash pasteurizer in seven to 10 seconds. The water in the jacket of the pasteurizer was heated by steam under a pressure of 10 pounds per square

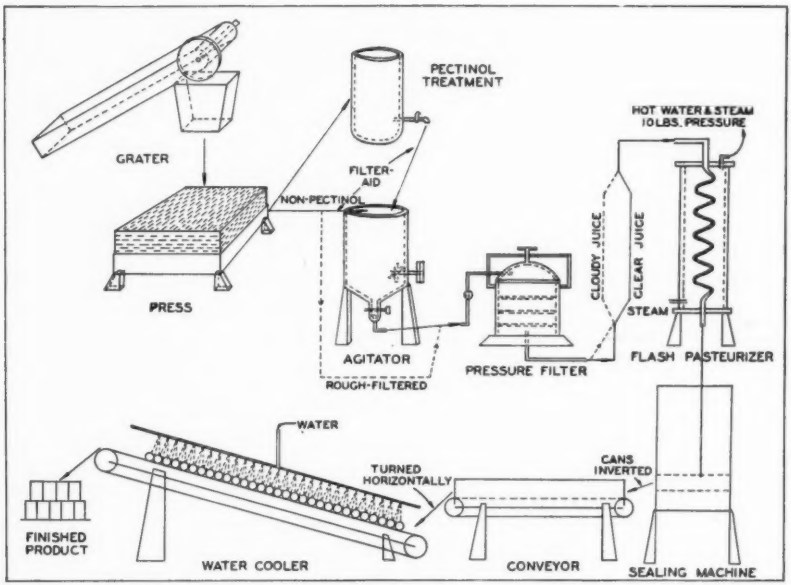

FIG. 1.

inch. The temperature of the water was automatically regulated by a thermostat which varied the amount of steam that was introduced. The juice passed through a flattened, spiral-shaped, metal coil made of block tin.

The cans and seals were washed and rinsed in hot water and steamed for three minutes immediately before use. The double enamel-lined cans were filled at once by hand and sealed rapidly by being passed through a power sealing machine. Upon being sealed, the cans were automatically inverted and transported over a conveyer for eight seconds to assure the sterilization of the seal. The cans were filled to the top with hot juice to prevent the retention of air in the can and hence, to prevent the growth of possibly noninactivated, aerobic microorganisms. In the samples of canned apple juice that were treated subsequently, a vacuum of 10 to 12 pounds per square inch was found. When the cans filled with hot juice reached the end of the conveyer described above, they were turned to a

horizontal position and rotated rapidly on an inclined belt under a spray of cold water; this procedure cooled the contents to a temperature of approximately 29.5°C.(85°F.) in about one minute.

EFFECTS OF APPLE WASHING UPON MICROORGANISM COUNT

The purposes of this part of the experiment were to determine the count of microorganisms on the apples themselves; the results obtained from washing in water; the effects of washing in HCl (1¼ per cent actual acid by weight) of the concentration ordinarily used in commercial apple washing, followed by rinsing in water; and the contamination from the air following washing.

The method of treatment was as follows: Six samples of apples were used in each of these studies, and the average count was taken after culturing on dextrose agar for 48 hours at 37 and 55°C.(98.6 and 131°F.) to determine the number of mesophilic and thermophilic organisms. The

TABLE 1

Number of Microorganisms on Washed, Unwashed, and Air-Contaminated Apples

Treatment	Organisms on 4 square inches	
	Mesophilic	Thermophilic
Unwashed apples	252	6
Washed with tap water, sampled immediately	13	6
Washed in 1¼%HCl by weight, sampled immediately	9	0
Washed with tap water, exposed to air for 5 minutes	18	6
Washed in 1¼% HCl by weight, exposed to air for 5 minutes	50	1

count of microorganisms on the apples was first determined; second, immediately after being washed in water; third, immediately after being washed in the HCl solution and rinsed in water; fourth, after being washed in water and allowed to remain exposed to the air for five minutes; and fifth, after being washed in the HCl solution and rinsed in water and allowed to remain exposed to the air for five minutes. Series four and five served as a means of determining the contamination by microorganisms from the air.

The microorganisms on the apples were counted by the following methods: (1) sterile agar discs, made by pouring 10 c.c. of sterile dextrose agar into sterile No. 2 cans and removing the hardened discs with a sterile spatula, were placed directly on the surfaces of the apples, removed, and incubated in sterile Petri plates; (2) quantitative dilutions from sterile swabs of four-square-inch surfaces of the apples were incubated on dextrose agar in Petri plates; and (3) Frost's "little plates" using dextrose agar were prepared and incubated. They were prepared in the manner described by Tanner for use in milk analysis. The first method was somewhat difficult to use; the third method was effective only when large numbers of organisms were involved and, therefore, did not prove effective in this experiment; the second method was superior to the first and third and, therefore, was used for most counts.

The results of this experiment (Table 1) show that the number of organisms on the apples is small. Of these organisms most are mesophilic,

and only a very small number are either thermophilic or facultative thermophilic. There is not an important difference between the count after washing the apples in dilute HCl and after washing them in water when the samples were taken immediately (13 mesophilic organisms per c.c. for water and nine for the HCl solution). When the samples were not taken immediately the count was larger, but the increase has no real bacteriological importance. The more rapid increase in surface contamination of the acid-washed apples standing in the air compared with the increase on similarly exposed water-washed apples, is not considered significant.

BACTERIOLOGY OF EQUIPMENT EMPLOYED IN PROCESSING

In this procedure those pieces of equipment which would be a probable source of microorganism contamination were analyzed bacteriologically (Fig. 1). The barrels held 10 gallons and had been lined with paraffin which was slightly marred and broken and provided an excellent seed bed for bacteria and other microorganisms. Two series of tests were made, one after rinsing with cold tap water (to prevent the destruction of the paraffin lining), the other after washing with calcium hypochlorite containing 550 parts per million of available chlorine (by analysis) and rinsing with cold tap water. The analysis consisted of swabbing four-square-inch areas and making quantitative Petri plates and Frost's "little plates," using dextrose agar as the medium. Six samples were used at 37 and 55°C. (98.6 and 131°F.). Readings were made at the end of 48 hours.

The cider receiving tank was examined in the same manner after a thorough washing with hot water. Samples were similarly obtained from the inner surfaces of the pressure filter after flushing thoroughly with hot water. The plates of this (laboratory) model are so constructed that thorough cleaning is not easily accomplished; particles of apple had accumulated, during the two months it had been used, in the crevices between the filter plate and its base.

The mixing tank, which is glass-lined, was sampled in almost the same manner as the receiving tank; however, only a warm water rinse was used.

Three series of tests were made on the cans. In one, the cans were rinsed only with warm water and swabbed. In the second experiment, the cans were immersed in a hypochlorite solution containing approximately 550 p.p.m. of available chlorine (by analysis), rinsed in warm water, inverted, and allowed to drain. A third sample of cans was rinsed with warm tap water, inverted, allowed to drain, thoroughly steamed for three to five minutes, and then sampled.

In the light of results in this series of experiments (Table 2) the author concludes that wooden equipment of the sort used in this experiment is an excellent source for the development and spread of microorganisms. This is so even after rinsing with a strong hypochlorite solution followed by water.

As for metal equipment, from the results obtained from analysis of the glass-lined mixing tank which has no seams or crevices, it is apparent that this type of equipment is ideal for juice processing.

The results obtained with the filter demonstrated that the aluminum surface cannot be looked upon as a sterilizing agent.

ANALYSIS OF JUICE DURING PROCESSING

A sample of juice from washed apples was taken from the receiving tank and cultured for 48 hours at 37 and 55°C.(98.6 and 131°F.) to determine the increase of organisms owing to contamination by the wooden receiving tank. After rough filtration through three layers of heavy filter paper, the juice was again sampled and cultured in order to show the decrease in microorganism count owing to the rough filtration. The juice was then pumped into wooden, paraffin-lined barrels and sampled again.

The next sample was taken after the juice had been clarified by filtering through a layer of diatomaceous earth. The clarified juice was pumped into wooden barrels and another sample was taken. Two samples of this clarified juice were stored at different temperatures, one at room temperature and the other at a temperature of 2.2 to 3.3°C.(36 to 38°F.). Twenty-four hours later the two lots of juice were analyzed to show the effects of the two different temperatures upon microorganism activity.

TABLE 2

Bacteriological Count on Surfaces of Equipment

Equipment	Organisms on 4 square inches	
	Mesophilic	Thermophilic
Barrels washed with cold water	Too many to count	18
Barrels washed with CaClOCl and rinsed with cold water.	104,800	15
Wooden receiving tank washed with hot water	2,000,000	21
Filter washed with hot water	Too many to count	0
Glass-lined mixing tank washed with water	3	1
Cans washed in warm water	3,077,000	2
Cans washed with CaClOCl and rinsed with water	2,320	0
Cans washed and steamed	50	0

A portion of the clarified juice which had been stored for 24 hours at 2.2 to 3.3°C.(36 to 38°F.) was flash pasteurized at 79.5°C.(175°F.) for approximately seven to 10 seconds. A sample of the juice was taken directly from the filling mechanism and allowed to cool slowly at room temperature to approximately 29.5°C.(85°F.) and then analyzed bacteriologically.

To determine the effect of spontaneous cooling upon thermophilic organisms, which it has been found are not inactivated in nonacid food unless the contents of the can are not cooled rapidly, a sample of this juice was taken directly from the filling mechanism and allowed to cool slowly at room temperature to approximately 29.5°C.(85°F.) and then analyzed bacteriologically.

A sample was taken from the juice as it was removed from the cooling machine after being rapidly cooled, in contrast to the preceding step. At the end of two months, a sample of the rapidly cooled juice was taken for analysis. This juice had been stored at room temperature, 21.1°C.(70°F.).

To show the effects of insanitation in processing upon the keeping qualities of apple juice, cans were not washed before filling. The juice

was pasteurized at 75.5°C.(168°F.) and filled directly into the unwashed cans.

It is evident from results of these experiments (Table 3) that the apple is not the major source of microorganisms. Most of the organisms found, however, were mesophilic. Although the original load of microorganisms on the apples was small, washing with water decreased the load appreciably. This decrease occurred in both mesophilic and thermophilic organisms (45 to 15 for the mesophilic and 15 to one for the thermophilic).

The juice was heavily seeded with microorganisms when it came in contact with the wooden receiving tank. Filtering the juice through heavy filter paper brought about a significant decrease in the bacterial load (from

TABLE 3

Microbiological Analysis During Processing

Stage of processing	Organisms on 4 square inches	
	Mesophilic	Thermophilic
Apples not washed	45	15
Apples washed in water and sampled immediately	15	1
	Organisms per c.c.	
Juice from wooden receiving tank	1,957,000	21
Juice rough filtered	540,540	5
Juice from barrels	1,788,000	....[1]
Filtered through diatomaceous earth	1,450	....[1]
Filtered juice from barrels	3,700	10
Clarified juice after 24 hours at room temperature	178,000	8
Clarified juice after 24 hours at 2.2 to 3.3°C.(36 to 38°F.)	103,000	5
Flash pasteurized juice slowly cooled	0	0
Flash pasteurized juice rapidly cooled	0	0
Rapidly cooled juice at end of 2 months' storage	0	0
Juice in unwashed cans, pasteurized at 75.5°C.(168°F.) after 2 weeks' storage at room temperature	Spoiled in cans[2]	

[1] Organisms not isolated or counted. [2] Type of organisms not determined.

1,957,000 organisms of the wooden receiving tank to 540,540 organisms after rough filtration).

A noticeable increase in microorganism number resulted, however, when the juice was pumped again into wooden barrels (from 540,540 to 1,788,000). This again illustrates the disadvantage of wooden containers in juice processing.

Filtration through diatomaceous earth brought about a pronounced and doubtless very important decrease in the microorganism number, as indicated by a reduction from 1,788,000 to 1,450 organisms per cubic centimeter. This amounts to 99.9 per cent. The number of organisms (1,450) present in the filtrate was undoubtedly due in part to contamination by the heavily seeded crevices of the filter located beneath the filter discs and directly in the path of the filtered juice as it passed to the effluent valve.

The results showing the effects of two different storage temperatures upon microorganism count indicate a rapid increase. Although there was not as great an increase in the microorganism number in the sample stored at cold-storage temperatures as there was in the sample stored at room

temperatures, nevertheless there was a definite increase in microorganisms in both cases (from 1,450 to 103,000 at cold-storage temperatures and to 178,000 at room temperatures).

Equally good results were secured from cooling the heated juice spontaneously as from cooling it rapidly, so that the necessity for rapid cooling to bring about inactivation of thermophilic organisms can be ruled out, provided the juice is pasteurized at a suitable temperature. A temperature of 79.5°C.(175°F.) for seven to 10 seconds under a steam pressure of 10 pounds per square inch was found to be sufficient under the conditions of this experiment; however, this did not hold true of juice in cans that had not been cleaned prior to filling.

EFFECTS OF FREEZING OF JUICE UPON MICROORGANISM COUNT

A study was undertaken to determine the effects of freezing upon the microflora of cider. A sample of juice from the cider receiving tank, containing 1,788,000 organisms per cubic centimeter, was frozen solid in a sterile gallon jug, sampled, and analyzed bacteriologically by means of the Frost's "little plates" and Petri plate methods at intervals of seven and 27 days. (This experiment is still in progress.)

TABLE 4

Effects of Freezing Upon Microorganism Count

Treatment	Organisms per c.c. mesophilic
Original juice from cider tank	1,788,000
Juice frozen for 7 days	426,000
Juice frozen for 27 days	1,320

In light of the results obtained from this experiment (Table 4) the microbiological flora decreases rapidly during freezing. The decrease in total number is especially great during the early period of freezing.[1]

SUMMARY

Processing apple juice is a relatively new and rapidly expanding industry; nevertheless, only a limited amount of research, especially of a bacterial nature, has been conducted in this field. These are the reasons for undertaking this series of studies.

The quantitative methods used in making the analyses consisted almost entirely of Petri plates and Frost's "little plates." In analyses where large numbers of organisms prevailed, the latter method proved to be more accurate and convenient.

The microbiological counts taken for apples after water washing and after HCl and water rinse showed only a slight difference; however, the apple was not an important source of microorganism contamination.

[1] The author found certain classes of organisms persisting longer than others. To be specific, in the original sample before freezing, rod-shaped bacteria and yeasts predominated, with mold spores and cocci less abundant. At the end of the freezing period of 27 days, however, the rod-shaped bacteria decreased in number, with a resulting higher ratio of cocci to the other flora. Only a few yeasts remained. This work is still in progress and the ultimate outcome will possibly be available at a later date.

Diatomaceous earth was much more effective than heavy filter paper in filtering out microorganisms. In this experiment diatomaceous earth filtered out 99.9 per cent of the microorganisms, compared with 72.3 per cent for the heavy filter paper.

Concerning the storage of apple juice, there was a rapid increase in the microorganism number, both at room and at cold-storage temperatures, 2.2 to 3.3°C.(36 to 38°F.), although there was a larger increase at the former temperature.

Although anaerobic, thermophilic organisms were found in the product, gradual cooling of the juice pasteurized at 79.5°C.(175°F.) for seven to 10 seconds did not reveal living organisms belonging to this class.

Under the conditions of this experiment, flash pasteurization of apple juice at 79.5°C.(175°F.) for seven to 10 seconds is sufficient for the preservation of this product.

Juice which was flash pasteurized at 75.5°C.(168°F.) for seven to 10 seconds and filled into unwashed cans spoiled after two weeks' storage at room temperature.

Freezing of juice was very effective in reducing the count of microorganisms, being 76 and 99.9 per cent for seven and 27 days, respectively.

The use of paraffin-lined equipment was not effective in preventing contamination by microorganisms.

Under the conditions of this experiment the use of hypochlorite solutions was not very effective in reducing the microorganism count, when compared with steaming the equipment. The microbial count of the cans after steaming was very small when compared with the initial number of microorganisms present in the cans before treatment.

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MEASUREMENT OF TEXTURE IN BAKED-POTATO TISSUE[1]

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The customary practice in determining the cooking quality of baked potatoes has been to estimate the texture by arbitrarily judging or scoring freshly cooked tubers. Sweetman (1936) has outlined criteria that distinguish four or five grades or degrees of texture. Other workers have indicated the differences by such vague descriptive terms as "very mealy," "slightly mealy," "slightly waxy," etc. The reliability and experimental error of these texture scores depend entirely on the experience and personal judgment of the observer. Even under the best conditions only four or five grades can be distinguished; and intermediate scores obviously include a large measure of uncertainty. The lack of an accurate method for measuring texture differences has severely handicapped investigational work on the cooking quality of potatoes. Some recent observations, however, seem to lead to a fairly accurate method for quantitatively measuring texture differences in baked-potato tissue.

It is apparent that the most important element of texture score is ease with which the cooked tissue mashes or disintegrates under pressure. This has led a number of authors to postulate the separation of the cells in mealy tissue and the adherence of the cells in waxy tissue. Sweetman (1936) and Personius and Sharp (1939) confirmed this suggestion by microscopic studies. Freeman (1941) reported that dried slices of baked potato tissue show differences that can be attributed to the separation and adherence of cells in mealy and waxy tissue, respectively. It was found that when mealy tissue was dried, a very porous mass resulted; but when waxy tissue was dried the slice became a very dense and horny vitreous sheet. The appearance of these samples was attributed to differences in cell separation because freshly baked tissue shrinks considerably on drying. If the cells have separated to any extent as in mealy tissue or if their bonding substance has become weakened the material shrinks away from these lines of separation leaving a network of pores between the dried cells. In waxy tissue, on the other hand, the cells adhere to one another so firmly that the material shrinks away from the outer surfaces of the sample. No pores are formed and the waxy tissue dries to a dense vitreous mass. Repeated observations have shown that the dried porous tissue came from the mealy portions of the baked tubers, while the dried, vitreous, nonporous substance came from the waxy or soggy samples. From these observations, it is believed that a quantitative measure of the porosity of the dried samples would serve as an index of the texture.

The best measure of the porosity probably would be the total surface area of the pores and internal air pockets. Since this would be difficult and tedious to measure in a large number of samples, a satisfactory ap-

[1] Contribution No. 434.

proximation was obtained by measuring the volume of the internal spaces. This would be only a first approximation since the pores and internal air spaces are by no means uniform. The internal air-space volume was determined directly by weighing the dried samples before and after the pores had been filled with an inert liquid. The difference in weight (i.e., the weight of a volume of toluene equal to the volume of the internal air space) was divided by the dry weight of the sample and multiplied by 100. This value, the percentage increase in weight of a dried sample owing to the absorbed toluene, is directly proportional to the pore volume and is called the texture index. This texture, or toluene, index was measured in a large number of samples and illustrative data are given in the description of the experimental procedure.

Since moisture content of raw tubers has long been recognized as a fairly reliable indicator of the texture of the baked tubers, a comparison of toluene index, judged texture scores, and moisture content was made on a series of 100 tubers. The moisture content was determined by direct analysis and also by the rapid specific gravity method proposed by Haddock and Blood (1939). The detailed data of these comparisons, attesting good agreement among these scoring methods, were so voluminous that only the correlation coefficients have been reported here.

EXPERIMENTAL WORK

Preparation of Samples: The samples were prepared as described by Freeman (1941). The skins were removed from freshly baked tubers, and slices three to five millimeters thick were taken from any or all parts of the tubers as desired. The slices were dried on glass plates in an air oven at 50°C.(122°F.), for several days. If dried at higher temperatures near 100°C.(212°F.), small blisters appeared under the dried surfaces. Since these blisters introduced large errors in the determination of pore volumes, the initial drying temperature should be kept well below 100°C. and preferably below 70°C.(158°F.). The partially dried chips were numbered with white ink and dried in a vacuum desiccator over phosphorus pentoxide to constant weight. The drying process was continued until successive weighings agreed within one or two milligrams.

Measurement of Porosity: The measurement of porosity was first carried out by a simple direct method. The dried chips were weighed, placed under toluene in a vacuum chamber, and the pressure was reduced by a water pump. Air was drawn from the samples until no more bubbles appeared. After atmospheric pressure had been restored, the saturated chips were removed, quickly wiped with a soft cloth to remove excess toluene from the surface, and immediately weighed in a tightly stoppered weighing bottle. The percentage increase in weight, proportional to the pore volume, was calculated and recorded as the toluene index. The chips were dried again and the procedure was repeated until reasonable agreement was obtained in successive determinations. Several hundred samples were treated in this manner and the few representative determinations given (Table 1) show an experimental error of about $\pm$ .5 per cent, with consistent reproducible values within this limit of error. The samples used in this experiment were also scored for texture according

to Sweetman (1936) and Freeman (1941). The agreement of the texture scores and the toluene indices shows clearly that both techniques measure the same characterisic. Further evidence on this point is given in a summary of some four hundred similar determinations (Table 2). In order to compare the toluene index with the judged scores the continuous series of toluene indices was arbitrarily divided into three groups that

TABLE 1

Successive Determinations of Texture Index on Dried Samples of Baked Potatoes

Dry weight of chip	Texture score, Sweetman (1936)	Texture index				Average
		1	2	3	4	
gm.		*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
2.944	5	13.9	9.9	11.8	13.5	12.3
2.434	5	10.6	9.7	9.4	9.4	9.8
2.159	5	8.9	8.4	7.7	8.5	8.4
2.339	4	6.5	6.6	6.3	6.5	6.5
2.339	3	5.3	5.2	5.3	5.3	5.3
2.148	3	4.9	4.8	5.0	5.1	4.9
2.143	2+	4.5	4.3	4.5	4.8	4.5
1.441	2+	4.0	4.3	4.0	4.2	4.1
1.773	2	3.1	2.9	3.0	3.1	3.0
1.952	2	2.6	3.0	3.0	2.9	2.9
3.565	1	1.3	1.2	1.6	1.8	1.5
1.999	1	1.3	1.3	1.3	1.5	1.5
1.698	1	0.7	0.6	1.0	.....	0.7
1.497	1	0.3	0.3	0.4	0.6	0.4

corresponded in range to the judged scores. The agreement is surprisingly good in view of the uncertainty of judged texture scores, especially in border line samples.

Two sources of error, however, were encountered in this direct method of measuring pore volumes. The removal of excess toluene by wiping the unsaturated chips immediately before weighing led to variations as high

TABLE 2

Comparison of Judged Texture Scores With Measured Texture Indices (Direct Weighing of Toluene Saturated Samples)

Texture index	Number of samples judged to have texture score:		
	Mealy	Intermediate	Waxy
pct.			
5.3 or greater	139	25	0
5.3 to 3.0	23	101	14
3.0 to 0.0	1	16	90

as ± .5 per cent, especially with inexperienced operators. Considerable practice was necessary to maintain a consistent technique and satisfactory checks. In many cases, it was apparent that pores were sealed off at the surface, thus preventing a complete saturation of the sample by the toluene. These difficulties result in a large experimental error in the first instance and low values in the second.

These errors were greatly reduced by modifications that also shortened the procedure. In this modified method the principle of Archimedes was employed to obtain the volume of the internal pore spaces. Three values were needed to calculate the toluene index: the weight of the chip in air; the weight of the chip suspended in toluene but buoyed up by the air trapped in the internal pores and air pockets; and the weight of the chip suspended in toluene but not buoyed up by entrapped air. The difference between the last two weights is the weight of toluene equal in volume to the internal air space. The first two values were determined by direct weighing on the analytical balance. The last value was calculated from the weight of the chip in air, the density of its dry matter, and the density of the toluene.

The samples were very carefully dried in a vacuum desiccator over phosphorus pentoxide until successive weighings agreed within one or two milligrams. The sample was then weighed in air and finally weighed immersed in a beaker of toluene by suspending the chip from one beam of the analytical balance by a fine wire and spring clip. This arrangement was similar to that of a Westphal balance. The level of liquid in the beaker must be kept constant since the rest point of this balance arrangement is affected by the amount of toluene displaced by the immersed chip and partially immersed wire. Obviously, care must be taken that the immersed chip does not touch the sides or bottom of the beaker.

The full weight of the sample (under toluene) not buoyed up by entrapped air was calculated by the following equation: $M_t = M_a \frac{(d - d_t)}{d}$ where M_a is the observed weight of the sample in air and M_t is the calculated weight in toluene with pores filled with toluene. The densities, d_t and d, are those of toluene and the dry matter of the chip, respectively. The density of the dry matter of the chip, d, was obtained by selecting five very carefully dried, waxy chips that had no pores or internal air spaces and weighing them under toluene. The densities, calculated in the usual way, were $1.533 \pm .003$. The assumption that no air pockets or pores were present was verified by subsequently evacuating these samples under toluene. No increase in weight was observed in any of the samples. The average value obtained for d was applied to all subsequent calculations on the assumption that the dry-matter densities of different potato samples do not differ by a significant amount. This assumption rests on two considerations: (1) the chief constituents of dried-potato tissue, starch, celluloses, and proteins all have densities very near 1.50; (2) the relative amounts of these constituents vary but little from one dried sample to another.

With these data, the toluene index was calculated by the following equation: toluene index $= \frac{M_t - M_T}{Ma} \cdot 100$ where M_a is the weight in air, M_T is the weight under toluene buoyed up by the air-filled pores, and M_t is the calculated weight under toluene with the pores filled with toluene.

Several hundred samples were run by this procedure with much more satisfactory results. The elimination of the errors encountered in the direct

method allowed values that could be reproduced with some degree of precision. The modifications reduced the experimental error about 10 times. In the direct method, texture indices ranged from .5 to 13 per cent with an estimated error of ± .4 to 1 per cent. In the modified procedure, the values ranged from .15 to 6.30 per cent with an estimated error of ± .05

TABLE 3

Successive Determinations of Texture Index on Dried Samples of Baked Potatoes

Dry weight of sample	Texture score, Sweetman (1936)	Texture index					Average
		1	2	3	4	5	
gm.		*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
2.418	4	5.65	5.50	5.45	5.40	5.45	5.50
1.875	4	4.70	4.75	4.75	4.70	4.65	4.70
3.052	4	4.10	4.25	4.25	4.15	4.25	4.20
3.074	4	4.10	4.25	4.25	4.20	4.20	4.20
2.647	4	3.45	3.40	3.55	3.50	3.50	3.50
1.433	3	3.20	3.10	3.15	3.15	3.10	3.15
1.840	3	3.00	3.10	3.10	3.10	2.95	3.05
1.382	3	2.90	2.90	3.00	2.90	.....	2.90
3.220	2	2.75	2.75	2.85	2.70	2.70	2.75
1.716	2	2.60	2.55	2.55	2.65	2.55	2.55
1.411	1+	1.20	1.30	1.20	1.20	1.20	1.25
1.433	1	1.05	1.05	1.10	1.05	1.00	1.05
2.005	1	0.70	0.80	0.80	0.90	0.80	0.80
1.253	1	0.40	0.55	0.55	0.50	0.45	0.50
1.433	1	0.15	0.15	0.00	0.25	0.15	0.15

to .10 per cent. Obviously both methods measure the same property and rank a series of samples in the same order. The few representative determinations (Table 3) demonstrate the greater accuracy of this method; a summary of the comparison of this method for texture index with the judged texture scores is shown (Table 4).

TABLE 4

Comparison of Judged Texture Scores With Measured Texture Indices (by Modified Procedure)

Toluene index	Number of samples judged to have texture score:		
	Mealy	Intermediate	Waxy
pct.			
6.3 to 3.5	106	22	0
3.5 to 1.5	24	127	18
1.5 to 0.0	3	37	110

Toluene Index and Moisture Content: Since it has been known for many years that the moisture content of raw tubers was a fairly reliable index of the texture that developed when the tuber was baked, it seemed desirable to test the correlation between toluene index and moisture content. In the experience of most workers, however, the moisture content was by no means perfectly correlated with judged texture scores, and excep-

tional results were commonly reported. Moisture determination on the raw tuber and subsequent baking tests cannot be easily run on the same tubers, hence direct comparisons were difficult. Furthermore, the determination of moisture contents has been too tedious for the routine examination of a large number of tubers. These difficulties were largely avoided by Haddock and Blood (1939), who suggested that the moisture content of the raw tubers could be rapidly measured by floating them in salt solutions of different specific gravities. The specific gravities of large numbers of tubers could be rapidly determined in this way and the tubers could be subsequently used for baking tests. The method is not entirely satisfactory, however, because individual tubers have been found to be far from uniform in the specific gravity (or moisture content) of their various regions. Tubers with an average specific gravity of 1.085 may actually vary from 1.070 to 1.100 in different parts of the same tuber. Consequently, the specific gravity of any given tuber is an average of widely differing parts. This means that only about four or five degrees of specific gravity are significantly different when interpreted as a texture score. The great advantages of the method, however, are the use of a measurable characteristic rather than an arbitrarily judged texture score and the rapidity with which a large number of samples can be roughly classified according to texture. The data of Haddock and Blood show very good agreement between five grades of judged texture scores and five divisions of specific gravities.

In this laboratory these different methods for estimating texture were carried out simultaneously on a lot of 100 tubers. The tubers were numbered and their specific gravities measured by immersion in salt solutions. After being baked for one hour at 218.3°C.(425°F.), the tubers were sliced and judged for texture score, according to Sweetman (1936). Alternate slices were dried for determination of toluene index while the remaining slices were immediately mixed and sampled for moisture determinations. The voluminous data were subjected to statistical analysis which indicated several definite relationships. Specific gravities ranged from 1.063 to 1.100. The moisture content of baked tissue ranged from 61 to 84 per cent. About 400 toluene indices ranged from .2 to 3.8 per cent.

The moisture contents of the baked tubers were significantly correlated with the specific gravities of the raw tuber, $r = .881 \pm .016$. This indicates that the same relative amount of moisture is lost from all tubers regardless of size or shape during the baking process. This situation was further clarified by the observation that nearly all the moisture lost during the baking process comes from the outer layers of the tuber. During baking periods of one, two, and three hours the interior flesh of tubers lost only .4, 1.0, and 1.3 per cent of its moisture, while the entire tuber lost 10, 20, and 40 per cent of its original weight. Thus it is apparent that the baking process progressively caused a complete desiccation of the outer layers, while the inner regions suffered practically no losses until the adjacent layers were almost completely dried.

The high correlation between moisture content, or specific gravity, and judged texture scores is a matter of common experience. The close cor-

respondence of judged texture scores to toluene or texture indices has been demonstrated above. In these experiments the average toluene index of each tuber (four or five samples per tuber) was compared with the specific gravity of that tuber. A coefficient of $r = .724 \pm .034$ was found for 100 tubers. The correlation of average toluene index of the tuber with the moisture content of the baked tissue yielded a coefficient of $.750 \pm .031$.

DISCUSSION

The relation of moisture content of texture and cooking quality has been amply demonstrated and reported in the literature. The relation of cell separation to texture and cooking quality has been reported and described in a qualitative way by means of photomicrographs etc., but no quantitative demonstration of this factor has been previously offered.

The above work clearly demonstrates that cell separation in baked-potato tissue is a factor of mealiness or texture and can be measured quantitatively. Thus it affords a new method for measuring texture, not as a vaguely descriptive score but as a precise quantitative measure.

Since moisture content and cell separation are factors in determining the texture of baked potatoes, the question arises as to whether there exists a causal relation between moisture content and cell separation. There are apparently two possibilities involved. Moisture content may be in some manner the cause, or at least a contributing factor, of cell separation and thus would show a high correlation with texture. Investigation of this point remains for further work. On the other hand, moisture content and cell separation may be two entirely independent factors of mealiness.

If this is true, a more comprehensive description of potato texture is possible. Mealiness or waxiness and sogginess or dryness could be independent criteria. A tuber might be waxy and dry instead of waxy and soggy or wet, as is usually the case. Dryness may not be an invariable corollary to mealiness. A baked tuber might possibly be mealy in texture but still wet and soggy. Specimens suggesting these descriptions have been observed but there is no definite evidence bearing on the question. Further consideration of this question may have some practical significance, however, since the different types of texture suggested above would better meet the culinary requirements encountered in such different processes as frying, baking, salads, and potato-chip manufacture.

SUMMARY

Evidence is offered for the existence of two factors influencing the texture and cooking quality of baked potatoes. These are original moisture content and the degree of, or tendency for, cell separation.

A method is outlined for a quantitative measurement of cell separation in baked samples. The close relation of this property to moisture content and judged texture scores has been clearly shown.

It has been pointed out that these two factors may or may not be independently related and that some practical aspects of cooking quality may be related to this question.

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A REVIEW OF THE MICROBIOLOGY OF COMMERCIAL SUGAR AND RELATED SWEETENING AGENTS

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Sweetening agents serve as one of the most important constituents of human diets, and their production and refining constitutes a major industry. The principal dietary sweetening agent is commercial sucrose or "sugar" derived from sugar cane and sugar beets. The latter industry is larger in the United States than the cane-sugar industry, but differences in the final products as sugar are practically indistinguishable insofar as use and marketing are concerned. Included as sweetening agents also are maple sugar and syrup, honey, corn sugar and syrup, sorghum, and cane molasses.

SUCROSE (CANE AND BEET SUGAR)

Within the boundaries of the United States over eight million tons of beet sugar and four million tons of cane sugar are produced. However, the cane sugar introduced from the Philippines, the West Indies, and South America makes the total amount of cane sugar consumed far greater than that of beet sugar.

The processes involved in the manufacture of cane and beet sugars are basically similar although the sources of the sucrose are of entirely different types. The problems dealing with the spoilage of sucrose in storage have been approached from the standpoint of both beet and cane sugars. The organisms involved and their control in the refineries as well as the raw-sugar factories in many instances are similar, although problems distinct to mill and to refineries are found in both the beet and cane industries. The questions of the microbiological deterioration of raw sugar, mill sanitation, and flora of fine sugar have been discussed in the literature, in the main interchangeably, with not too much appreciation of the fact that mill and factory microbiological problems, although closely related to, may be entirely distinct from refinery and refined-sugar problems. Consequently this discussion will approach the published literature primarily from the standpoint of the types of organisms encountered in each type of sweetening agent.

In the probable loss of sucrose in the raw juice during manufacture, insofar as bacteria are concerned, two types of organisms have been particularly incriminated. The one most commonly encountered and earliest described in connection with sugar spoilage is the "frog spawn" fungus or *Leuconostoc*. The other group is the sporeforming organism, little being known of the nature of changes it produces. In the case of *Leuconostoc* it is generally noted that one of the evidences of this spoilage may be a production of slime generally composed of levan or dextran. For this reason, comments on the historical development of bacterial spoil-

age are closely intertwined with discussions dealing with sliminess in sugar and juices.

In connection with the whole subject of organisms in sugar, both beet and cane, the organisms are practically all derived from the sugar houses or during the early processes of extraction. In the case of cane, Millstein, Tobin, and McCleskey (1940) have shown that cane syrups may contain large numbers of organisms. The raw syrups contain large numbers of mesophiles which are removed by the clarifiers, while the number of thermophiles in the raw juice is relatively low with an increase as the crystallization process proceeds. Van der Linden (1916) felt, however, that significant bacterial spoilage of sugar in storage was due to local infection after the sugar was packed. He studied a deteriorated pile of sacked raw sugar stored in high (80 per cent) humidity.

Leuconostoc and Related Types: Insofar as sugar deterioration is concerned Browne (1918) gives one of the best modern discussions and historical developments, while Kircher (1839) was probably among the first to find organisms in slimy beet juice. He reported that these organisms would form slime when grown in sucrose solutions. Feltz (1874) found the accumulation of slime in presses sufficiently serious in certain cases as to cause many clarifying difficulties. From such observations one would assume that they may have been studying representatives of what is known today as the genus *Leuconostoc.* Brüning (1857) found that a slimy product from the fermentation of milk seemed to be identical in many respects with the slimy growth encountered in beet-sugar juice. Further study by this investigator indicated the slime to be largely arabinic acid. Others who also studied the composition of this same slime, reported that it contained an acid of cellulose and metapectinic acid. These observations stimulated a series of articles by Scheibler (1868, 1869 a, b, c, 1870, 1873, 1874, 1875, 1878, 1880, 1884, 1887, 1895) and Jubert (1874), which involved a discussion as to the cause or origin of slime in deteriorating sugars and raw juices. Scheibler believed that the slime was a mixture of cell protoplasm in the case of beet juice and though inert bacteria were present they played no part in the production of the slime. Jubert disagreed with Scheibler inasmuch as he found that slime production could be checked by phenol or heating the juice to 90°C.(194°F.) or above, and from such observations concluded that a living organism was associated with the development of this slime. Further studies by Scheibler strengthened his contention that slime was purely a mixture of plasma from the best cells, although he did report that beet juice became slimy when allowed to stand. He developed a number of chemical methods for the analyses of the slime and on a basis of these methods reported its composition in detail. Somewhat later, Scheibler was found to conclude that *Zoogloea terme* might cause the formation of slime under certain conditions. Although he found a large number of bacteria and yeasts in the slime, he maintained the opinion that the presence of these living organisms was more or less incidental. In his later chemical investigations he separated from the slime a gum-like substance which he recognized as an anhydrid of glucose and called it a dextran.

Both Scheibler and Jubert stimulated further work on the cause of slime formation in sugar juices. Mendes (1874) agreed with Jubert as he examined slime under a microscope and found it contained large numbers of small globular granules, in from two to six pairs, and thought them to be causative organisms. Feltz (1876a,b) confirmed in part Jubert's and Mendes' observations, reporting that bacteria cause the production of slime; but Borschtschoff (1876) agreed with Scheibler and concluded that slime was but plasma or pectin secreted by the beets.

Durin (1876) studied the chemical composition of the slime and reported that there may be more than one type of slime present and that the differentiation of these types may explain in part why the earlier workers could not agree on observation substantiated by Votocek and Sebor (1899). Bauer (1882) reported a series of analyses and gave more definite information as to the chemical composition of these slimes in sugar solutions.

Cienkowski (1878) was probably the first investigator to study sugar slimes from a microbiological standpoint, and as a result he described a species which he named *Ascococcus mesenteroides* as the cause of the "frog spawn" slime in sugar solutions. He found that the slime was not soluble in the usual cellulose solvents and concluded that no cellulose was present. His results supported the conclusions of Jubert and Mendes and probably for the first time definitely established a specific organism as the cause of slime production in sugar. Van Tieghem (1878) studied Cienkowski's organism and called it *Leuconostoc mesenteroides* inasmuch as it resembled a species of *Nostoc*, a blue-green alga. Following these investigations a large number of workers began to study organisms in fermenting and slimy sugar solutions, particularly beet syrup. Béchamp (1881) and Daeumichen (1890) both found organisms in beet juice which would ferment mannitol and form slime from sucrose. The former called this slime "viscose" while the latter thought the slime was a dextran, probably closely related to mucin.

In light of more modern developments regarding these organisms, it is interesting to note that Schardinger (1890) determined that the acid produced by these slime-forming types which decompose sugar was optically different from the lactic acid produced by other and closely related types of cocci.

Liesenberg and Zopf (1892) became interested in the detailed descriptions of these slime organisms, studied the cultural characteristics of the *Leuconostoc* types, and found that gum was produced most actively in slightly alkaline media. The latter studied three cultures of *Leuconostoc* isolated from German and Javanese sugar mills and found that the organisms would resist 80°C.(176°F.) for five minutes. Liesenberg was probably the first to cultivate in pure culture the slime-producing organism. He used the Petri plate method and thought the capsule which was produced was not a true capsule but rather a product of metabolism. He also discovered that if he heated the slime to 75°C.(167°F.) for 15 minutes to kill the organisms outside of the slime, those engulfed in the slime were found to have resisted. Although the organisms were believed to

produce slime more rapidly in alkaline sucrose gelatin, the development of slime could not be used as a character for differentiation.

Several *Leuconostoc* types were immediately found in slimy sugar conditions by a number of workers; among others was *Leuconostoc Lagerheimii*, described by Ludwig (1892). Hucker and Pederson (1930) discuss these *Leuconostoc* types more completely from the standpoint of their cultural characteristics and taxonomic relations. Ward and Green (1900) isolated an organism from sugar solutions which formed gum from sucrose but concluded that it grew symbiotically with yeast and that such gum showed it was not a dextran. These observations are of particular interest in light of the more recent conclusion that the *Leuconostoc* types require some growth accessory substance similar to yeast extract for maximum growth production. From a taxonomic point of view Maassen (1904) believed that these *Leuconostoc* types were sufficiently like the streptococci to be included in that particular genus, and more recently Voille (1922) stated that the organism responsible for gum formation in sugar was very similar to *Streptococcus lacticus*. He found large capsules formed which caused the organisms to adhere in large masses and he also determined that the composition of the medium very largely controlled the formation of gum and that the gum was formed only from sucrose. In his experiments he determined that the gum formed under these conditions could be readily precipitated by alcohol.

Zettnow (1907), further studying the cocci associated with slime production, named two gas-producing types, *Leuconostoc opalanitza* and *Leuconostoc aller*. He concluded that these organisms would not produce slime in dextrose solutions and failed to find any types as heat resistant as those earlier reported. Schöne (1904) described two coccus types from slime in sugar syrups and reported that the first type formed acetic acid, lactic acid, and gas but did not produce slime in beet juice; whereas the second group, similar in most respects, formed slime in milk and in beet juice and produced acetic acid, dextro-lactic acid, and gas.

Müller-Thurgau (1908) found a coccus in wine which produced no slime in the wine but when grown in sucrose solutions produced a gum-like substance as well as mannitol, and on further studies he determined that this particular organism produced gas. More recently Hucker and Pedersen (1930), in studying the entire group of gas-producing, streptococcus-like types, concluded that species of the genus *Leuconostoc* differ primarily from streptococci in producing carbon dioxide and levolactic acid from the fermentation of glucose. Of the three *Leuconostoc* species described by these workers two were found to produce slime in sucrose solutions while a third, although a gas-producing type, did not utilize sucrose and therefore could not produce slime under any of the conditions studied. These organisms were found to be common in fermenting sauerkraut and other types of vegetables. They are also considered of the aroma type in dairy starters and similar products and are probably one of the contributing factors to slime production in sugar syrups.

Slime production and the deterioration of sugar, both from the standpoint of sugar crystals and juice, have been found to be caused by a number of organisms other than those of the *Leuconostoc* type. Bräutigam

(1891) described an organism which he called *Micrococcus gelatinogenus* which produced a dextran causing sliminess in sugar. No cultures of this organism are available and thus its relation to the *Leuconostoc* types must remain for the present unknown.

Sporeforming Types: That sporeforming bacteria are associated with the deterioration of both sugar crystals and juices is reflected by the large number of references to sporeforming types causing sugar spoilage. Although Payen (1851) suggested that microorganisms may be responsible for sugar deterioration and later Kramer (1889) described *Bacillus viscosus sacchari*, it was not until the work of Owen (1911, 1914, 1916, 1918, 1925, 1932) that the sporebearing bacteria were definitely associated with deterioration of sugar. He concluded that the destruction of sucrose, particularly raw sugar cane in storage, was due to levanase produced in turn by members of the sporebearing types of the potato bacillus group. The spores of these organisms were found to be unusually resistant. The organisms produced a gum which was found to be produced at a maximum in an alkaline medium. Owen went so far as to conclude that all slime-forming bacteria were of the sporebearing type.

Van der Bijl (1920), in studying the resistance of certain of the sporebearing types found in sugar solutions, determined that all of these types survived 100°C.(212°F.) for two hours or more and were not destroyed through 30 minutes' exposure in one to 50 formalin solution or one per cent solution of sodium fluoride. He was of the opinion that low temperatures and low relative humidity in stored sugars, together with maintenance of proper sanitary conditions in mills, would reduce the incidence of spoilage of raw sugar by bacteria.

Greig-Smith (1902) described another sporeforming type, *Bacillus levaniformans*, which deteriorated sugar, owing to its ability to form levan from sucrose.

Miscellaneous Bacteria: Ritsert (1891) studied the gum found in cane-sugar juices. He reported that such gum was produced by a pleomorphic type of organisms which he called *Bacterium gummosum*. He described it as being Gram-negative and producing spores. Happ (1893) also studied a closely related organism which he called *Micrococcus gummosus*, which produced a slime and formed butyric and lactic acid as fermentation end products. Glaser (1895) isolated a rod-like type to which he gave the name *Bacterium gelatinosum betae*, but Owen (1911) reported this particular organism produced alcohol rather than lactic acid. The characteristics and relationships of all of these slime-producing, rod types have been summarized by Perquin (1940).

That any of these organisms might play an important part in sugar determinations was questioned by Browne (1918) who stated that microorganisms could not survive for any periods in stored sugar. Samples held for two years, for example, might be found to be sterile. Keller (1939) outlines in this connection an excellent review of the prevailing opinions and literature on the factors influencing the keeping quality of raw sugars. Although he concludes that microorganisms are the final cause, contributing factors are concerned with moisture relations during storage.

Yeasts: A limited amount of investigation has been carried on insofar as any relationship is concerned between the presence of yeast and the spoilage of sugar. Owen (1918) found that *Saccharomyces soffi* could be isolated from sugar but only with difficulty. These isolations were possible only if the medium used was of the same density as the syrup from which the organisms were to be isolated. Jörgensen (1911), in studying several samples of refined sugars, reported torulae to be present.

Molds: Although the spoilage of sugar has long been a problem through loss of sucrose by inversion, it was not until Shorey (1898) made a study of the problem, that molds were reported to be associated with this particular type of sugar deterioration. He concluded that the organism most likely responsible for the inversion during storage was *Penicillium glaucum.* In studying the origin of this mold in sugar, he was of the opinion that spores gained entrance to the sugar in the centrifugals and believed that there was a definite correlation between deterioration and those districts in which a large amount of dust came in contact with the sugar. He believed that sterilization with dry steam was the most effective procedure in keeping the molds at a minimum. Kamerling (1899) studied a number of cane sugars from Java and found that the fungus flora was much similar to certain species of *Penicillium.* Of the 20 varieties which he isolated, 19 were of this type and he concluded that the deterioration of raw sugar was at least initiated by fungi. Somewhat later Greig-Smith (1902) confirmed very largely the work of Shorey that sugar deterioration was largely brought about by molds and that these molds gained entrance to the raw sugar through unsanitary and unsatisfactory mill conditions. He found *Aspergillus glaucus* in small-grain, moist, refined sugar but concluded that in addition to these molds certain sporeforming types of bacteria were very significant in sugar deterioration. He thought that an organism to which he gave the name *Bacillus levaniformans* may deteriorate sugar on account of its ability to form levan from sucrose. Mold hyphae were found in samples of stored sugar by Townsend (1904), and he reported that it sporulated in saturated sugar solutions within six to eight days. However, his findings have remained unconfirmed. Schöne (1906, 1908, 1911) also found penicillia, particularly *Penicillium glaucus* and *Penicillium mucor,* in sugar and believed them to be extremely important as factors in the inversion of sucrose. His studies of pure cultures indicated that they may have a very definite effect upon the acidity of sugar solutions.

Somewhat later Scott (1912) found both penicillia and aspergilli on the surface of sugar liquors. He was of the opinion, however, that various types of sugar, particularly with respect to their origin, responded differently to the action of these molds. He included in his studies low Brazilian, Peruvian, Jamaican, and Javanese sugars.

The activity of fungi in certain sugar syrups in Javanese factories was studied by Amons (1916, 1917, 1921), while Browne (1915-1939) made a number of chemical observations concerning sugar deterioration and discussed this deterioration from the standpoint of moisture, abnormal fermentations, etc. He found monillia as well as certain penicillia and felt that they were important factors in the fermentation of sugar syrups

but also discovered that certain torulae had considerable inverting power. Amons continued his investigations, using special types of media, and was able to isolate from deteriorating sugar *Aspergillus rhizopi* and *Penicillium glaucum*. The latter was observed in active form through finding mycelial threads in moist sugar. Sugar which had been sterilized with absolute alcohol was inoculated with mold spores and it was noted that sterile sugar controls absorbed no moisture after 35 days, while the inoculated sugar absorbed relatively large amounts of water. Upon these facts it was concluded that molds may make an appreciable growth in sugar and probably invert the sucrose with the hydroscopic reducing sugars absorbing moisture. Although these organisms were thought to be the cause of deterioration of sugar, Amons concluded that more than one type of organism was the general cause of the loss of sucrose in storage.

Some of the most extensive pieces of investigation on the deterioration of cane sugar have been carried on by Owen (1918). His studies have been based largely upon cane-sugar mills and refineries in the United States and more particularly mills in Louisiana. As a result of his investigations, Owen has concluded that fungi may bring about a marked deterioration of sugar through inversion, and he is of the opinion that such organisms are the most significant of all the groups of microorganisms found in the sugar industry owing to the decided inverting power of fungi and the possibility of their growth in highly concentrated solutions of sucrose. He found that a 69° Brix solution was beyond the concentration in which either bacteria or torulae could grow although there were certain fungus spores which could resist this concentration of sugar. Grove (1918) found that *Penicillium glaucum* could grow in a 65-per cent sucrose solution, although when this concentration was raised to 70 per cent only a slight growth was observed after one month. Kopeloff and Kopeloff (1919) and later Kopeloff and Byall (1920) reported a long list of mold fungi in sugar which were found to be present mostly in the spore stage. These investigators also studied the invertase activity of *Penicillium expansum* and found that this particular organism would continue its inverting power over a range of concentrations from 10 to 70 per cent of sugar. Beyond this concentration no activity was found. The maximum activity was found in concentrations of 50 to 60 per cent. Amons (1921) again reported *Penicillium glaucum* as particularly common in sugars in Java but *Penicillium luteum* and *Penicillium rubrum* as being rather rare in occurrence in spoiling sugars. Kopeloff, Perkins, and Welcome (1921) identified levan formed by enzymes produced by *Aspergillus sydowi* and analyzed a number of sugar solutions of varying concentrations for sucrose and gum. They believed the gum to be formed by the levanase elaborated by the mold spores from invert sugar. In the absence of glucose little gum was found.

More recently Van der Bijl (1920) studied the deterioration of South African sugars and made special investigation of four varieties of molds: *Aspergillus, Stemphylium, Sterigmalocystis,* and *Hormodendron,* in the latter genus the species *H. eladosporoides.* With the exception of *Stemphylium,* all grew in solutions of 63° Brix which was the highest concentration of sugar tested. He reported that boiling the sugar solutions for 15 minutes

entirely killed the molds and also that certain substances could be added to the mill juice to inhibit the growth of these spores. If to 50 c.c. of mill juice were added either .27 gram of sodium chloride, .5 gram of ammonium fluoride, four c.c. of a five-per cent sodium bisulphide solution, 12 c.c. of a .5-per cent commercial formalin and six c.c. of a two-per cent chloride of lime solution containing 16 per cent of available chlorine, or calcium hydroxide containing five per cent calcium oxide, the growth of any of these four types of molds would be inhibited.

Control of Sugar Spoilage: Considerable effort has been expended in an endeavor to control the deterioration of sugar and particularly the formation of slime in sugar mills. Kamerling (1900), Friedrich (1906), Schulz (1903), Strohmer (1905), Simpson (1907), and Spencer (1929) have recommended the use of formaldehyde in various concentrations and in various forms to prevent the spoilage of raw sugars. Two and one-half to five grams of formaldehyde per 100 kilos have been recommended to keep down fermentation in the diffusion batteries of beet-sugar factories by certain workers, while others have found that in order to prevent fermentation of mill juice in the factories, one part of formaldehyde to 20,000 parts of juice was necessary. All in all, the use of formaldehyde has not been too successful in controlling fermentation.

Other investigators, particularly Saillard (1900), Effront (1908), and Harloff and Schmidt (1913), have recommended fluorides in the preventing of sucrose inversion. These investigators have reported that fluorides of approximately one-per cent strength would serve as an active germicide in sugar solutions, particularly in diffusion batteries in beet-sugar factories. Saillard (1900) used sodium and calcium bisulphide in the diffusion batteries, but Effront found that certain yeasts could be trained to grow in high concentrations of fluorides.

More recently Owen (1926) perfected a unique process for preserving sugar through the inoculation of sugar with yeast, this yeast capable of growing in the high tensity of a molasses film surrounding the sugar crystals. The yeast is a non-sucrose fermenter but forms carbon dioxide from levulose. This yeast would saturate the film with carbon dioxide and prevent mold growth on the sugar crystals. The loss of the levulose reduces the hydroscopic nature of the sugar, thereby bringing the inoculated syrup nearer within the safety limits. Other methods of controlling sliminess have been suggested, one of which, by Winter (1893), confirmed the use of ammonium lactate and calcium hydroxide, although the latter was found somewhat inefficient for the control of the *Leuconostoc* type of slime.

Tempany and D'Emmerez de Charmey (1922) reported an extensive study of Mauritius sugar and believed torulae to be the important spoilage organisms and concluded that it was impossible to keep sugar sterile and to prevent all deterioration. However, Owen (1932) and Owen and Mobley (1932) discuss the refining process of cane sugar and emphasize during the refining process the importance of maintaining sanitary conditions along with efficient filtration to keep thermophilic anaerobes at a minimum in the refined sugar. Earlier Gayon (1880) had also suggested factors other than chemical disinfectants in controlling raw-sugar fermentation and

believed that the changes in stored sugar were due primarily to unfavorable moisture and temperature conditions. In this connection Diago [1] suggested in a more concrete form the importance of the amount of moisture in sugar by concluding that of two sugars of the same water content the purer will deteriorate more rapidly, and on this fact suggested the so-called

safety factor, i.e., $\frac{\text{Relation between \% water}}{\text{non-sugar}}$ .25.

A number of investigators have reported general studies on the flora of sugars and reviewed the literature pertaining to various types of spoilage under a large number of conditions. Durin (1876), Lewton-Brain and Deerr (1908), Deerr and Norris (1908), Blake (1918), Church (1921), and Bell and Staunton (1935) have all summarized the work in this particular field.

Anaerobic Thermophiles in Cane Sugar: Insofar as most of the earlier studies on the microbiological content of sugar are concerned the major stress has been placed upon the number of organisms present and their relation to the spoilage of sweetened foods rather than the effect of such organisms on the deterioration of the sugar itself, either raw or refined. Weinzirl (1927), in studying candy spoilage, found that 85 per cent of 33 sugars used in candy manufacture contained anaerobes. He thought them to be of no great significance so far as the sugar was concerned but definitely showed for the first time that the flora of the sugar may affect its contaminating value as a sweetening agent. Little concerted effort in the earlier days had been given to the possibility of sugar being a source of contamination for foods to which it was added either before or subsequent to processing. The results of Barlow [2] (1913) indicated that the organisms in sugar may be of significance insofar as spoilage of canned foods is concerned, but it was not until later that Cameron, Williams, and Thompson (1928) studied the causes of spoilage of canned corn and other nonacid foods and found the spoilage to be due to thermophilic anaerobes and traced the source of these organisms to the sugar used as a sweetening agent.

Following this work, Cameron and Williams (1928) and Cameron (1936) reported the technical details of a bacteriological study regarding thermophilic sporeformers in sugars. In the investigations of the thermophilic, sporeforming content of sugar, Cameron and his associates pointed out that there are three groups of sporebearing bacteria of major importance in the spoilage of nonacid canned vegetables: (a) thermophilic anaerobes which may be classed as causing flat sours and which produce acid but no gas, (b) thermophilic anaerobes which form acid and gas (the gas is made up entirely of carbon and hydrogen but no hydrogen sulfide), and (c) thermophilic anaerobes which produce a gas that is chiefly hydrogen sulfide with very small traces of acid.

The work of Cameron (1930), Cameron and Yesair (1931), Duval (1931), Cameron and Bigelow (1931), and Homans (1933) largely estab-

[1] Unpublished.

[2] Unpublished master of science thesis, University of Illinois, Urbana, Ill.

lished the fact that, unless carefully handled during refining, a granulated cane sugar may contain large numbers of these organisms, particularly of the flat-sour type. This work was in part confirmed by James (1928a,b). That these organisms were associated primarily with refineries was discussed by Owen and Mobley (1932). The beet-sugar industry has been confronted with a parallel problem, as shown by Calton (1936) and Hall (1939). The flat-sour type of thermophilic anaerobes, according to the workers, appears to be the most commonly encountered in all of this general group of organisms although there is a wide variation as regards the number of spores in individual samples. In refined cane sugar from one to 500 spores per gram of the flat-sour type were found. The gas-producing types, both of the group producing hydrogen sulfide and of the group not producing it, were found not to be as common as the flat-sour types.

The spores of these organisms in all of the groups were found to be especially resistant to heating. Under practical conditions 95 per cent of the flat-sour spores in certain sugars resisted 121.1°C.(250°F.) for 70 minutes, and 50 per cent of these spores still remained after subjection to the same temperature for 90 minutes. The relation of these organisms to the spoilage of nonacid foods is rather significant. This significance, according to the above workers, is not due primarily to direct contamination of the foods by addition of spores in the syrups made from sugars with a high content of sporebearing types, but rather from contamination of the cannery by syrup solutions and the subsequent infection from these secondary sources of the nonacid foods being processed.

In order to eliminate, if possible, the sporebearing thermophiles from refined sugar, investigations were made of possible sources of these thermophilic types. As a result, it was found that there was a larger number of thermophilic anaerobes in refined sugar than in raw sugar as it reached the refinery. However, the types were very similar and it was concluded that the refineries were originally contaminated by the organisms brought to the refinery in the raw sugar. During the refining process the bacteriological aspects appear to pass through three distinct phases or stages. A large number of anaerobic sporeforming types were found in the refining process prior to filtration. Subsequent to filtration there is a distinct increase in the anaerobes, particularly the flat-sour types, and this final sugar contamination appears to be greater than that in the raw sugar being used at the same time. By extra filtration and crystallization, however, it was found that the number of these organisms could be reduced to a practical minimum.

With these results at hand the National Canners laboratory has set up definite standards for the approval of various batches or lots of sugar to be used in canning. These sugars, upon analysis, shall not contain more than 75 spores of the flat-sour types per 10 grams. In like manner, a maximum has been established for the number of gas-producing, anaerobic, thermophilic spores which are allowable.

The above investigators have carried on a certain amount of work on the source of these thermophilic anaerobes, as have James (1928b) and Owen (1932), the latter pointing out that thermophilic anaerobes, particularly of the flat-sour type, must indicate a contamination through raw

sugar since he was unable to find organisms of the flat-sour type in any of the nine soil samples which he studied. However, of nine samples of soil collected from fertilized fields, six contained gas-producing anaerobes and six contained thermophilic gas-producers of the hydrogen-sulfide type.

Co-operation between the canning industry and the sugar refineries has brought about a decrease in the number of thermophilic, anaerobic types found in refined sugar. At the moment canneries are using only sugar which carries a guarantee from their refineries that it meets the standards of the National Canners Laboratory, thus avoiding a large percentage of the spoilage which had been heretofore experienced.

More recently Dekker (1934) has found flat-sour and sulphur types in Java refined sugar, while Ingersoll (1930) believes the significance of thermophiles in sugar to be underestimated. As to effective methods of controlling the presence of thermophiles in sugar, all are agreed that proper sanitary measures in refineries are the most practical and effective. Hall and Keane (1939) studied the use of radiant energy (2337 Å) and found nearly one-half of the species in sugar would be killed if sugar in the granulator of a beet-sugar factory was properly irradiated. However, this procedure has not received widespread adoption. Plant practice of repeated crystal washing has resulted in a reduction of the spores since Hall and James (1936) found the organisms in crystal sugar to be on the surface of the crystals and removable by repeated washings.

MOLASSES

Various types of molasses have been studied in themselves but little work has been published on the microbiological conditions. Hall, James, and Nelson (1936), in studying certain cane syrups, particularly Barbados molasses, isolated a total of 11 cultures from two samples of this type of molasses and concluded that this material has as its predominant flora two groups of yeast, *Zygosaccharomyces nussbaumeri* and *Z. major*. Gas production or frothy germination in stored tanks of mill molasses is encountered many times. Gillet (1917) has reported similar foaming in massecuites in beet and Lafar (1914) in cane sugar factories. This gas may be caused, as suggested by Browne (1919, 1929), by the possible decomposition of calcium gluconate, with the production of carbon dioxide. The gluconate may be formed from the excess lime failing neutralization. Carbon dioxide may be produced from amino acids in the presence of reducing sugars.

However, there have been a number of studies and observations on the possible cause of this gas production. Ashby (1909), Herzfeld (1913), Laxa (1905), Claassen (1910), and others believed the phenomena to be entirely or largely due to microbiological activity; Lafar (1914), in addition to Browne, also presented strong arguments that molasses foaming in storage is due to some spontaneous chemical action. The history of the development of these chemical considerations of foaming of molasses on storage has been well outlined by Browne (1929, 1939).

Dodson and Browne (1903) studied the microbiological flora of Louisiana cane syrups. However, in certain special cases particular methods of molasses or syrup handling may enhance the growth of specific types of

organisms with a resulting characteristic flavor. This is especially true of syrups and molasses produced in Barbados. Hall, James, and Nelson (1936) found this flavor to be due primarily to the production of alcohol, furfural, etc., owing to the action of certain yeasts and bacteria. The handling of this Barbados molasses, according to Browne (1919), Tempary (1913), and Watt and Tempary (1905), is somewhat different from that of molasses or syrups in the commonly encountered mills. The syrup is handled so as to reduce crystallization of the sugar and little effort is made to check the growth of organisms, with a consequence that a slow fermentation takes place resulting in the flavors and characteristics associated with Barbados molasses. Rum manufactured from this type of molasses, according to Allen (1906), is dependent also upon this carbon fermentation for its particular qualities.

LACTOSE

Although until recently lactose has not been considered as a sweetening agent, Kühl (1912, 1913) studied the bacterial content of lactose and concluded that if stored lactose contained only a small amount of nitrogenous substances, no deterioration was found. However, if nitrogenous substances were present to promote growth, lactose could be deteriorated by bacteria.

SORGHUM

The sorghum industry is not localized in any particular section and, inasmuch as sorghum is not manufactured in centralized plants, the problem of its spoilage has not become apparent or acute. However, Bryan (1912) reports on the spoilage of sorghum, particularly its variation in acidity and also the development of certain fermentation by-products. He found that either heating or the addition of lime appeared to control definitely this deterioration.

MAPLE SUGAR AND MAPLE SYRUP

Maple sugar and maple syrup are of minor significance from the standpoint of world production of sugar and have little in common with cane and beet sugar as a general sweetening agent; however, as a specialized sweetener they are of considerable significance. A million and a half tons of cane sugar are produced in the United States annually. Approximately three million gallons of maple syrup are produced, while eight times this amount of canned syrup is produced a year. Geographically maple syrup is confined to certain districts where the sugar maple is found, chiefly in the northeastern part of the United States and certain sections of Canada.

Microbiological problems have always confronted the maple sugar producer but these problems have been due to handling of the syrup rather than to any normal microbiological flora of the original sap. Edson (1910a,b) reported that the original sap in the vascular bundles was sterile but became contaminated in the tap bales and by the spout and buckets, causing objectionable and abnormal conditions known as "green sap," "moldy sap," or "stringy sap." A study of a series of these abnormal saps and syrups showed that such conditions are due to various microorganisms. The green sap had been thought to be caused by the

effect of swelling buds in the spring and therefore is known as "buddy sap." Edson found, however, that certain species of *Pseudomonas* could be isolated from these buddy saps and, when reinoculated into maple sap, produced the characteristics of buddy sap. Furthermore, he also found that the time of the season did not affect the sap. Many workers observed that early in the season the sap and resulting syrup were clear but as the season advanced, the sap and syrup became green, milky, or cloudy. Many thought this to be due not only to the swelling buds but to physiological changes in the sap or possibly to auto-enzymatic changes. Edson showed that in practically all cases these changes were due to contaminated spouts and utensils as the season advanced. Early experiments with milky sap indicated that organisms isolated from milky sap and syrup would reproduce this condition when reinoculated into normal sap or syrup. In addition, an organism from stringy sap when inoculated into maple sap produced a characteristic milky condition. The results of this preliminary investigation are of particular interest inasmuch as Edson not only definitely associated microorganisms with changes in the sap and syrup but noted that certain bacteria grew more rapidly in syrups inoculated with yeasts. This observation, in light of the recent work of Hucker and Pederson (1930), is of particular interest inasmuch as these workers have shown the need for the presence of yeast or extracts of yeast in the medium so that the *Leuconostoc* types may develop. These types would be expected to be present in milky and cloudy sap and syrup in light of results in the study of cane and beet sugar syrups. However, Edson did not succeed in isolating *Leuconostoc* from maple syrups, probably owing to the nature of the media employed, i.e., nutrient agar containing peptone and meat extract and a synthetic medium, neither of which would support the growth of *Leuconostoc*.

In the preliminary work Edson also reported that the reducing sugar content was consistently decreased in the syrups inoculated with bacteria, over the controls, while the yeast and mold inoculated syrups gave a reduced sucrose but increased reducing sugar content.

Later, Edson, Jones, and Carpenter (1912) studied abnormal maple syrups more in detail, and in general reported that although in the early part of the season only a few microorganisms were present, these types were largely bacteria, while later in the season the yeast-like types predominated. This was contrary to the findings of Edson (1910b) who reported yeasts in the first runs to be replaced by bacteria later in the season. Edson, Jones, and Carpenter (1912) found in some samples, on the average, from 300,000 to 140,000,000 organisms per cubic centimeter using nutrient agar as the plating medium; while less than 1,000 per cubic centimeter were found in carefully selected normal syrups; and, with few exceptions, less than 10,000 per cubic centimeter in commercial sweet syrup. In studying abnormal syrups these workers reported that stringy and milky sap and syrup were due to *Bacillus aceris*, a Gram-negative, gas-producing rod which was originally isolated from stringy syrup and which produced this characteristic when reinoculated into normal syrup. It was also suggested that *Micrococcus roseus* was the cause of pink sap and syrup, while the green sap and syrup were due to a variety of green fluo-

rescent types, chief among which was a new species given the name of *Bacillus parallelus*. This name was derived from the characteristic arrangement of the individual cells in the capsules. However, all in all, a large number of the green fluorescent type was isolated and a study of 42 strains indicated that nine were nonliquefiers, 32 liquefiers, and one of doubtful relationship to the fluorescent group. The nonliquefiers, however, evidenced some liquefaction when held from two to five months. These authors concluded that all the fluorescent types found in green sap and syrup, with the exception of *Bacillus parallelus*, should be considered as varieties of *Pseudomonas fluorescens*.

Fabian and Buskirk (1935), in studying ropiness in maple syrup, concluded that *Aerobacter aerogenes* was one of the prime causes. The addition of an amount of acetic acid of approximately the acidity found in fermented sap did not influence the consistency of the evaporated sap while a similar amount of lactic acid did influence the consistency. Neutralizing the acidity of fermented sap reduced somewhat the ropy condition of the concentrated sap.

That mold will develop on canned commercial maple syrup was noted by Heald and Pool (1908), who found a mold on the surface of such syrup, considered it a new species and gave it the name *Torula saccharina*. It was considered to be closely related to *T. sacchari-lactis* but differed only in average size and color of the spores. This mold was found to spread over the surface of the syrup in the can in 10 to 15 days when held at 28°C.(82.4°F.), depending upon the concentration of sugar in the syrup. Molds have been shown by Eschenhagan (1889) to grow in substances with a high osmotic equivalent. Thiele (1896) found that certain species of *Penicillium* would grow on solutions with as high as 70 per cent sugar or 25 per cent glycerol. These concentrations were believed to be the maximum for mold growth until Heald and Pool reported that *T. saccharina* had an optimum 45 per cent concentration of sugar for growth but would grow in solutions with as high as 75 per cent sugar.

Fellers (1933) studied maple sugar which had been stored in tubs and concluded that the off-flavors in the sugar and also in the pure and blended maple syrups made from maple saps, were due to molds, principally *Aspergillus*. The molds produce invertase, inverting the sugar and thus increasing its hydroscopic properties.

Fabian and Hall (1933) studied 19 yeasts from fermented Vermont maple syrup which were classified into seven groups. These yeasts in maple syrup required 60°C.(140°F.) for five to 10 minutes to kill the vegetative cells, and five minutes at 75°C.(167°F.) to kill the ascospores. They believed some of these forms responsible for fermenting maple syrup.

The whole subject of the spoilage of maple syrup has been well reviewed by Bryan (1910).

In light of more recent investigations on cane and beet sugar solutions and syrups it appears that more investigations are in order on the flora of abnormal maple syrup and saps, particularly in cases where the abnormality is characterized as being cloudy or milky, a characteristic which might be induced by some of the *Leuconostoc* types which have

been overlooked since the media used in the earlier investigations do not promote the growth of this group.

HONEY

Although honey has a high sugar content its spoilage under certain conditions long has been a problem with bee keepers. Various types of organisms have been thought responsible and many microbiological studies of honey have been completed, one of the most comprehensive of which has been that of Fehlmann (1911).

Yeasts: The yeasts have been thought most commonly to be associated with fermenting honey. Klöcker (1905-07) isolated *Zygosaccharomyces prioranus* from the bee and assumed that this organism was the principal factor in honey spoilage. White (1906) isolated and studied three yeasts from honey: *Saccharomyces F* from the intestines of bees, where he thought it to be frequent and common; *S. roseus* from comb; and *Saccharomyces G* from a diseased brood. Later Nussbaumer (1910) found several yeasts in honey and suggested in order to prevent fermentation that honey be heated for 30 minutes to 70°C.(158°F.).

Many investigators, Richter (1912), Lindner (1920), and Ewert (1925), studied the yeasts found in honey. Richter (1920) described *Zygosaccharomyces mellis acidi* and considered it as the cause of spoilage, and Lindner concluded that these yeasts spend the winter in the bumble bee. That yeasts are the chief causes of honey spoilage was further substantiated when Marvin (1928, 1930) found that the undesirable flavors found in fermenting honey are due largely to alcohol, nonvolatile acids, and CO_2. To avoid that spoilage, heating to 71°C. and sealing while hot was suggested. However, the color may be injured unless the heated honey is promptly cooled. The above products were not found if the honey was unheated and held at 11°C.(51.8°F.) or below.

That yeasts and other microorganisms will grow in honey, and more particularly in crystallizing honey, remained a problem for some time until Marvin pointed out the fact that crystallization no doubt brought about conditions favorable to yeast growth through lowering the actual sugar content of the water films around the crystals. Not only Marvin but later Marvin *et al.* (1931) stated that during crystallization the water solvent for the dextrose is retained as the liquid part of the honey and thus crystallization dilutes or increases the moisture content. However, Lochhead and McMaster (1931) found in 191 Canadian honey samples an average of 1,000 yeasts of sugar-tolerant types per gram and concluded that adaptability of the yeast cells to the increased sugar content and not the water increase through crystallization, is the cause of honey fermentation.

These suggestions as to the reasons why honey will ferment even with its high concentrations of sugar have stimulated many discussions regarding yeasts which not only survive but develop under such conditions. Fabian and Quinet (1928) believed that the types found in honey become acclimated to increased concentrations of sugar following contamination and grow on the surface. Of the honey yeasts they described five groups: *Zygosaccharomyces japonicus*, *Z. barkeri*, *Z. mellis*, *Z. prioranus*, and

Torulae mellis. The cells of these groups were much more resistant in honey than in broth cultures. The vegetative cells of the four *Zygosaccharomyces* were killed in broth when heated for 10 minutes at 50°C. (122°F.) but required for killing in honey five minutes at 60°C.(140°F.). The *Torula* species required 60°C. for 10 minutes in honey and 50°C. for 15 minutes in broth to eliminate all the vegetative cells.

To control honey spoilage, Lochhead *et al.* (1929, 1930, 1931) approached the problem from the possibility of the elimination of the yeast through cleanliness and sanitation as well as the addition of preservatives. In the latter case it was found that sodium benzoate would suppress the growth of the yeasts in fermenting honey, and in addition a study of 34 different flowers visited by bees disclosed 11 different honey yeasts, two identical with those found in fermenting honey. Some of these same strains were also found in hives and in soil close to the hives.

Bacteria Algae: Organisms other than yeasts have been found in honey and thought to be responsible for spoilage. A green alga, *Pleurococcus vulgaris*, has been found in Swiss honey which was able to withstand high concentrations. This group of organisms has not been commonly found in honey but certain types of bacteria have been isolated and studied. White (1921) made a cultural study of 1,200 bees and observed microscopically preparations of the intestinal contents of a large number and concluded that the flora of the bee intestine was made up of four types of bacteria. These types were all acid but not gas producers. He believed the intestinal flora to be destructive, constant, and simple. Schweizer (1928) concluded that French and Swiss honey was practically free from bacteria. Sartory and Moreau (1911) found yeasts in honey and also sporebearing types of bacteria, staphylococci, and molds. Of particular interest was a sporeformer thought similar to *Bacillus flavus* or *B. luteus* which produced a pigment slightly soluble in absolute alcohol at 30°C. (86°F.) and which turned yellow to brown upon the addition of alkali and back to yellow when acidified.

No reports have been made on the presence of pathogenic organisms in honey nor has honey been incriminated in connection with human disease. Sackett (1919), using pure extracted alpha honey, found that *Bacterium typhi* remained alive for 48 hours only, and if the honey was diluted, the time of survival was shorter.

NECTAR

The origin of yeasts in honey has long been an object of study. Whether by contamination from the outside through various indirect sources or more directly from the flower nectar has not been definitely established. Boutroux (1881, 1884) discussed yeasts in nectar while Root (1910) made the interesting comment that certain bees may have died as a result of feeding upon fermented nectar. That yeasts and other organisms are common in the nectar of most flowers was proposed by Hilkenbach (1911), Stoltz (1911), and Betts (1920). Yeasts in nectar, upon further study, were found to be characteristic of a type in that most nectar yeasts were observed to be in cross forms, and such types were named *Anthomyces reukaufii* by Grüss (1917) in honor of Reukauf (1911)

who reported the presence of such forms. Grüss found also that this type of yeast was not only present in nectar but in the proboscis of the bumble bee. Schuster and Vladimir (1913), in a more comprehensive study of 32 different plants, found bacteria and yeasts in the nectar and grouped the latter into three groups. Three yeasts with the characteristic cross form were thought by Hautmann (1924) to be due to a lack of nitrogen and sugar in the nectar and he succeeded in changing these yeasts to normal forms by changing the amount of nitrogen and sugar.

In a most extensive study, Schoellhorn (1919) distinguished between the yeasts found in nectar and those found in dust and also observed that greenhouse flowers contained nectar generally free from yeasts or other microorganisms.

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GAS PRODUCTION IN STORAGE MOLASSES

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Foaming or frothy fermentation of storage molasses is no new phenomenon in the sugar industry. For a number of years this fermentation has been known to occur not only in molasses vats and storage tanks but in other types of similar concentrated sugar materials. The literature, reviewed by Hucker and Pederson[1] (1942), contains many references to gas production in molasses in all degrees of severity. In certain instances the reaction has proceeded to the point of explosive foaming with the residual material becoming a charred mass.

Opinions of those who have investigated this problem are varied as to the possible causes, both primary and secondary. Certain investigators have concluded that it is primarily due to the action of microorganisms with the production of carbon dioxide, while others attribute frothy fermentation to chemical reactions between certain of the normal constituents of the molasses. The literature contains much data in support of both of these points of view. However, more mature opinion on the subject substantiates the belief that the primary cause of this frothy fermentation is chemical and not due in the main to the action of microorganisms.

A series of samples from three large sugar mills were secured from the West Indies during the grinding season of 1939 and shipped in refrigerated five-gallon tins to the laboratory for subsequent study, while additional studies were made at the mills on freshly secured samples.

It was the object of this investigation to determine the factors which control the frothy fermentation of molasses on storage and to learn whether or not practical control measures could be instigated under storage conditions.

CARBON DIOXIDE PRODUCTION IN MOLASSES

Effect of Hydrogen-Ion Concentration on Glucose-Amino Acid Reaction: One of the most feasible explanations as to the cause of gas production in storage molasses is a reaction between the reducing sugar and the amino acids normally present. Mixtures of various concentrations of glycine and glucose were made in Eldredge tubes, and incubated for two weeks to determine the amount of carbon dioxide produced. Large amounts (Table 1) of carbon dioxide were produced under these conditions indicating a definite chemical reaction between glycine and glucose with the production of gas. It was found that by raising the H-ion concentration, the amount of carbon dioxide evolved by the reaction between glycine and glucose could be materially reduced. As a result of these observations, a series of molasses samples was studied for gas production under factory conditions. Four gallons each of the various molasses sam-

[1] Hucker, G. J., and Pederson, C. S., 1942. A review of the microbiology of commercial sugar and related sweetening agents. Food Research 7, 459-480.

TABLE 1

Carbon Dioxide Production in Eldredge Tubes From One Part Glycine and Four Parts Glucose Incubated at 45°C.(113°F.) for 14 Days

Eldredge tube No.	Phenol added	H-ion concentration	Carbon dioxide produced from 20 c.c. in 14 days
		pH	*mgm.*
1	—	5.6	8.8
2	+	5.6	8.6
3	—	4.5	6.4
4	+	4.5	6.6
5	—	4.0	....
6	+	4.0	5.1
7	—	3.5	4.0
8	+	3.5	3.5

TABLE 2

Carbon Dioxide Production by Four Gallons of Molasses (Plant A) Incubated for 14 Days at 40°C.(104°F.) in Sealed Five-Gallon Containers

Sample		Treatment	Carbon dioxide produced as indicated by pressure developed in sealed container	
No.	Source		Visual examination	Pressure readings
				lb./sq. in.
1	From final centrifugal	No treatment	+	1.0
2	Wharf storage	No treatment	0	0.0
3	Dock	No treatment	0	0.0
4	From final centrifugal	pH 4.6	+++	5.5
5	From final centrifugal	pH 4.0	+++	4.5
6	From final centrifugal	pH 3.7	+	0.0
7	From final centrifugal	pH 3.0	+++	Broken
1A	From final centrifugal	.7% phenol	++	2.5
2A	Wharf storage	.7% phenol	+++	2.5
3A	Dock	.7% phenol	+±	0.5
4A	From final centrifugal	pH 4.6, .7% phenol	++	2.5
5A	From final centrifugal	pH 4.0, .7 %phenol	+++	4.2
6A	From final centrifugal	pH 3.7, .7% phenol	+++	3.0
7A	From final centrifugal	pH 3.0, .7% phenol	+(?)	0.0

TABLE 3

Effect of H-ion Concentration on Carbon Dioxide Production by Foaming Molasses (Plant A) in Eldredge Tubes

Eldredge tube No.	Basic medium	Sucrose in basic medium	H-ion concentration	Temperature of incubation for 2 days	Carbon dioxide produced
		pct.	*pH*	*°C.*	*mgm.*
1	Yeast extract, glucose	10	7.0	65	12.2
2	Yeast extract, glucose	10	7.0	45	56.3
3	Yeast extract, glucose	1	7.0	65	28.0
4	Yeast extract, glucose	1	7.0	45	56.3
5	Yeast extract, glucose	10	6.5	65	8.6
6	Yeast extract, glucose	10	6.5	45	56.3
7	Yeast extract, glucose	1	6.5	65	.....
8	Yeast extract, glucose	1	6.5	45	52.4
9	Malt extract	10	5.2	65	5.5
10	Malt extract	10	5.2	45	51.7
11	Malt extract	30	5.2	65	4.1
12	Malt extract	30	5.2	45	56.3

ples were placed in five-gallon sealed containers. One series of seven containers was treated with phenol, the other remained untreated. The H-ion concentration of these 14 containers was raised from normal, as found in the final molasses when the samples were collected through various mill stages, to pH 3. The containers were placed at approximately 40°C.(104°F.) for 14 days.

Upon examination by determining the amount of pressure developed through the evolution of carbon dioxide in these cans, it was found that raising the H-ion concentration under actual factory conditions did not control the production of gas from the molasses. Neither was any effect evidenced through the addition of phenol (Table 2) to retard the growth of microorganisms.

Significance of Microorganisms: It is generally known that yeasts may grow and produce carbon dioxide in highly concentrated sugar solutions. Samples of foaming molasses (Table 3) were inoculated into yeast extract-glucose and malt extract media in Eldredge tubes to determine whether or not gas-producing organisms were present in the foaming molasses as might be indicated by the production of carbon dioxide.

The results (Table 3) indicated that the organisms found in foaming molasses will produce carbon dioxide when such molasses is inoculated directly into the proper nutrient medium. It was noted that these organisms produced their greatest amount of carbon dioxide at 45°C.(113°F.); and when the incubation temperature was raised to 65°C.(149°F.), the amount of carbon dioxide, produced probably by microorganisms, obviously was materially reduced.

In addition, it was noted that an increase in H-ion concentration did not materially affect the growth of these organisms as reflected by the carbon dioxide produced.

Cultures were isolated from the samples showing excessive foaming and were grown in molasses, both diluted and undiluted, and the amount of carbon dioxide produced was determined. Certain of these cultures did not produce carbon dioxide in undiluted molasses but did produce increasing amounts if the molasses was diluted. Under factory storage conditions their growth may take place in the condensation water on the surface of the storage tanks and, once acclimated to the higher concentrations of sugar, produce carbon dioxide from the undiluted molasses.

Further experiments (Table 4), using molasses from different sources as a basic medium, gave the first indication that the different types of molasses vary in their potentiality for the production of carbon dioxide. It was found that a sporebearing rod (SF8) isolated from the pipe line between the plant (A) and the storage tanks, when inoculated into molasses and incubated under the proper conditions, would produce carbon dioxide when incubated at 45°C. It became obvious, however, that uninoculated molasses, even though previously sterilized, when incubated at high temperatures produced relatively high amounts of carbon dioxide (Table 5). This was particularly true when the temperature of incubation was raised as high as 65°C. which is above the growth temperatures of the organisms isolated from the foaming molasses. Certain thermophilic sporeformers were isolated which grew at 55°C.(131°F.).

TABLE 4

Carbon Dioxide Production by Molasses in Eldredge Tubes

Sample tested and used as inoculum	Basic molasses used as a medium	Temperature of incubation	Carbon dioxide produced in 10 c.c. molasses, incubated 3 days
		°C.	mgm.
Foaming molasses, Plant A	Plant A, Vat No. 1	45	22.8
Foaming molasses, Plant A	Plant A, Vat No. 1	65	.....
Gas-producing sporeformer from pipe line, Plant A (SF8)	Plant A, Vat No. 1	45	24.0
Gas-producing sporeformer from pipe line, Plant A (SF8)	Plant A, Vat No. 1	65	.....
Control, no inoculum	Plant A, Vat No. 1	45	18.5
Control, no inoculum	Plant A, Vat No. 1	65	49.5
Foaming molasses, Plant A	American Sugar Refining Co.	45	17.8
Foaming molasses, Plant A	American Sugar Refining Co.	65	55.4+
Gas-producing sporeformer from pipe line, Plant A (SF8)	American Sugar Refining Co.	45	14.5
Gas-producing sporeformer from pipe line, Plant A (SF8)	American Sugar Refining Co.	65	44.6
Control, no inoculum	American Sugar Refining Co.	45	17.2
Control, no inoculum	American Sugar Refining Co.	65	40.8
Foaming molasses, Plant A	"Grandmothers Old Fashioned" molasses	45	10.3
Foaming molasses, Plant A	"Grandmothers Old Fashioned" molasses	65	32.1
Gas-producing sporeformer from pipe line, Plant A (SF8)	"Grandmothers Old Fashioned" molasses	45	14.5
Gas-producing sporeformer from pipe line, Plant A (SF8)	"Grandmothers Old Fashioned" molasses	65	27.5
Control, no inoculum	"Grandmothers Old Fashioned" molasses	45	10.3
Control, no inoculum	"Grandmothers Old Fashioned" molasses	65	22.2
Gas-producing sporeformer from pipe line, Plant A (SF8)	Plant A, Vat No. 1	45	17.3
Gas-producing sporeformer from pipe line, Plant A (SF8)	Plant A, Vat No. 1	65	.....
Gas-producing sporeformer from Plant C storage (SFe3)	Plant A, Vat No. 1	45	20.0
Gas-producing sporeformer from Plant C storage (SFe3)	Plant A, Vat No. 1	65	55.6
Control, no inoculum	Plant A, Vat No. 1	45	19.1
Control, no inoculum	Plant A, Vat No. 1	65	55.6
Gas-producing sporeformer from pipe line, Plant A (SF8)	American Sugar Refining Co.	45	15.0
Gas-producing sporeformer from pipe line, Plant A (SF8)	American Sugar Refining Co.	65	47.3
Gas-producing sporeformer from Plant C storage (SFe3)	American Sugar Refining Co.	45	14.1
Gas-producing sporeformer from Plant C storage (SFe3)	American Sugar Refining Co.	65	50.4
Control, no inoculum	American Sugar Refining Co.	45	13.9
Control, no inoculum	American Sugar Refining Co.	65	43.8
Gas-producing sporeformer from pipe line, Plant A (SF8)	"Grandmothers Old Fashioned" molasses	45	13.2
Gas-producing sporeformer from pipe line, Plant A (SF8)	"Grandmothers Old Fashioned" molasses	65	31.5
Gas-producing sporeformer from Plant C storage (SFe3)	"Grandmothers Old Fashioned" molasses	45	12.8
Gas-producing sporeformer from Plant C storage (SFe3)	"Grandmothers Old Fashioned" molasses	65	29.9
Control, no inoculum	"Grandmothers Old Fashioned" molasses	45	15.0
Control, no inoculum	"Grandmothers Old Fashioned" molasses	65	30.8

Processing samples were secured at various steps in the plant as well as from shipments of foaming molasses to determine the possible source of contamination by gas-producing organisms. These samples were inoculated into a tryptone-glucose medium in Eldredge tubes incubated at 25°C. (77°F.), and the amount of carbon dioxide was determined. This temperature of incubation is below the range affecting chemical reactions in molasses but within the range of biological activity. It was found that the organisms which may produce gas in molasses were present not only in the storage tank and service pipe line from the mill to the storage tanks but were also in the molasses when it left the final centrifugal spout in the factory (Table 5). This observation may vary with the sample studied (Table 7), as a further observation of processing samples would indicate that the microorganisms in the molasses are to be found in various processing stages in the factory. Unsterilized samples secured from various points in the factory and diluted also produced carbon dioxide under optimum conditions.

Effect of Temperature on Carbon Dioxide Production in Molasses: It was earlier noted that the amount of carbon dioxide produced in molasses (Table 4) was directly dependent upon the temperature at which the molasses was held. During the various inoculation experiments it was found that the molasses which was held at the higher temperature, even though uninoculated, produced a greater amount of carbon dioxide under controlled conditions than similar samples held at lower temperatures. With this in mind, two series of molasses samples were studied to determine the possibility of the effect of temperature directly upon carbon dioxide production.

Earlier in these experiments it was found advisable to discontinue the use of 100°C.(212°F.) as an incubation temperature inasmuch as such large amounts of carbon dioxide were produced that either the apparatus was broken or difficulty was encountered in keeping the apparatus completely closed.

The results of these temperature experiments (Table 6, Figs. 1, 2) indicate that there is a very direct relationship between the temperature at which the molasses is held and the amount of carbon dioxide produced. These experiments also indicate that the microorganisms present may play a very minor, if any, role in gas production in molasses. It will be noted that the amount of carbon dioxide produced increases directly with the increase in temperature when the molasses being observed has been previously sterilized. It can be noted also that the amount of carbon dioxide produced in temperatures ranging from 25 to 65°C.(77 to 149°F.) in the unsterilized molasses in one sample (commercial molasses) is slightly greater than the carbon dioxide produced at the same range of temperature in the sterilized molasses. No definite information is available on the history and treatment of this sample.

These data would indicate that probably a spontaneous chemical reaction plays the most important part in the production of carbon dioxide in the range of temperatures at which molasses is commonly stored. However, under certain conditions and with special types of molasses it is possible that at approximately 40°C.(104°F.) a portion of the carbon

TABLE 5

Carbon Dioxide Production by Molasses When Inoculated Into a Tryptone Glucose Broth

Plant A	Carbon dioxide produced in Eldredge tubes				
	1st trial, 6/27/39	2nd trial, 7/13/39	3rd trial, 7/20/39	4th trial, 7/25/39	5th trial, 8/31/39
	Sample inoculated into 20 c.c. medium; incubated at 25°C. (77°F.) for 3 days[1]	Sample inoculated into 10 c.e. medium; incubated at 25°C. for 4 days	Sample inoculated into 10 c.e. medium; incubated at 25°C. for 2 days	Sample inoculated into 10 c.e. medium; incubated at 25°C. for 3 days	Sample inoculated into 20 c.c. medium; incubated at 37°C. (98.6°F.) for 1½ days
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Service pipe line	0.0	1.2	.....	37.2+	58.4+
Bottom storage tank	35.5	36.2+	23.4	37.2+	58.4+
Top storage tank	31.1	36.2+	.....	37.2+	55.2
Final centrifugal spout	30.1	10.5	.....	14.0	56.8
Molasses boat	.....	32.7	.....	37.2+	38.8
Final centrifugal	0.0	4.0	.....	37.2+	49.3
Factory pump	0.0	36.2+	.....	8.6	51.2
Wharf pump	0.0	36.2+	18.7	37.2+	58.4+
Final centrifugal (Plant B)	0.0	.....	.....	.....	.....
Final molasses (Plant B)	0.0	.....	.....	.....	.....
Control 1	0.0	0.0	0.0	0.0	0.0
Control 2	.....	.....	0.0	.....	.....
Control 3	.....	.....	0.0	2.6	0.0
Control 4	.....	.....	.....	2.1	0.0

[1] Medium for first five trials adjusted to pH 7.

TABLE 6

Effect of Temperature on Carbon Dioxide Production by Sterilized and Unsterilized Molasses

Temperature of incubation	Carbon dioxide produced				
	Plant A (one-day incubation)		Plant B (four-day incubation)	Commercial molasses (two-day incubation)	
	Molasses *not* sterilized prior to tests	Molasses sterilized prior to tests	Molasses *not* sterilized prior to tests	Molasses *not* sterilized prior to tests	Molasses sterilized prior to tests
°C.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1.1	0.9	6.2	0	0.8	2.4
7.2	1.1	4.2	0	2.2	2.2
18.0	2.4	7.0	0	5.1	2.0
21.0	1.3	4.2	0	4.4	3.1
25.0	3.3	6.8	0	6.2	3.5
30.0	9.7	7.7	0	9.7	3.7
37.0	4.6	8.4	0	13.0	6.4
45.0	8.1	9.7	9	.....	7.7
65.0	17.4	13.4	87	20.0	14.3
80.0	32.1	49.3	207	34.8	31.9

dioxide produced is brought about by the activity of microorganisms, but as the temperature increases the part played by microorganisms decreases. It should also be noted that the zone of increased carbon dioxide production in these samples, showing possible effects of microorganism activity, is from 25 to 65°C., corresponding to the temperature range of growth of the gas-producing sporeformers isolated from foaming molasses. In all cases, however, the temperature of storage is generally more important than the activity of microorganisms.

Further experiments on the effect of temperature on production of gas by molasses indicated that the amount of carbon dioxide produced by

TABLE 7

Carbon Dioxide Production in Eldredge Tubes by Samples Taken From Various Stages in Process in Plant A

Sample		Carbon dioxide produced by one part sample and 100 parts sterile water incubated at 30°C. (86°F.) for 2 days
No.	Origin	
		mgm.
1	Molasses from pump at factory storage tank	33.6
2	Molasses (C) from canal between centrifugal and weigh tank	18.9
3	Massecuite (C) from crystallizer after cooling	27.7
4	Molasses (B) from storage tank	0.0
5	Molasses (MA) from storage tank	27.0
6	Molasses (A) from storage tank	33.2
9	Water from wash bucket in front of "C" centrifuge	22.4
10	Molasses from surface of final storage tank	42.6
11	Raw sugar	0.0

molasses follows a logarithmic curve (Fig. 1) when held at increasing temperatures. Also, there appears to be a critical temperature at which point gas production is sufficiently pronounced as to produce active and nearly explosive foaming of the molasses. This critical temperature, in the range of approximately 40°C., may vary several degrees depending upon the type and source of molasses being studied. It will be noted that this critical temperature of 40°C. is within the range of higher air temperatures encountered in the geographical location from which the foaming samples were obtained.

VISIBLE FOAMING IN MOLASSES

It must be considered that carbon dioxide may be produced in molasses and certain amounts remain in solution and produce no appreciable evidence of foam. The preceding experiments were continued to determine if laboratory results could be duplicated under conditions more nearly approaching molasses storage conditions. In this series of observations the amount of foam appearing on the surface of the molasses was taken as the index of gas production rather than an actual determination of the amount of carbon dioxide produced. The factors found to control carbon dioxide production under laboratory conditions were studied with visible

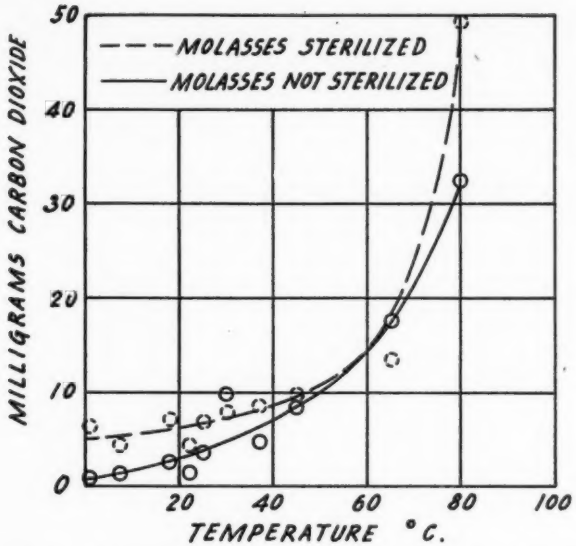

FIG. 1. Relation of temperature to carbon dioxide production in storage (Plant A, RW2) molasses.

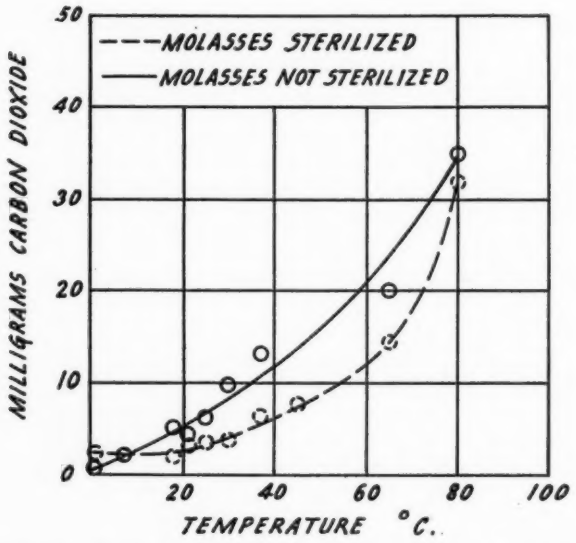

FIG. 2. Relation of temperature to carbon dioxide production in commercial canned molasses.

foaming as an index rather than carbon dioxide production. The undiluted samples were placed in glass cylinders and the amount of foam determined as increase over the original volume.

Effect of Temperature: Based on the above suggestive results a detailed study was made on the effect of holding temperature upon visible foaming of molasses. The amount of foam as per cent of original volume was determined. A sample was obtained directly from the final (Plant A) centrifugal and held at 27, 37, 45, 65, and 80°C.(80.6, 98.6, 113, 149, and 176°F.) (Table 8). The amount of foam was found to increase with the increase in temperature.

A similar series of samples from the wharf storage tank showed comparable results. In the case of the sample held at higher temperatures the foam was produced quickly, and once the mass of carbon dioxide had escaped from it, the molasses assumed more nearly its original level. The critical temperature at which the increased foaming was observed was between 37 and 45°C. At the latter temperature it was noted that at the end of three days there was 33 per cent increase in volume of the molasses owing to the excessive foam. This foaming increased to over 90 per cent at the end of about two weeks, i.e., the volume of the molasses doubled owing to the production of carbon dioxide.

At the higher temperatures large amounts of carbon dioxide were produced in the first 24 hours. In many cases the volume of the molasses was more than doubled. In these instances the viscosity of the molasses was materially reduced, owing to the higher temperatures, and the escape of the gas was thereby facilitated.

In studying additional samples (Plant B) the results appear to be somewhat different from those obtained above. The volume of foam produced from molasses from another plant (Plant B—Second Series, Table 8) was much less even at comparable temperatures. It was noted also that these samples when held at 37°C. for two weeks produced only eight per cent foam, while the first samples (Plant A) held for the same length of time produced four times this amount or approximately 34 per cent (Table 8). When the second sample (Plant B) was held at 45°C. for two weeks, only 46 per cent foam was produced in contrast to 84 per cent foam in those cases when samples from the first plant (A) were held at 45°C. for two weeks. These results would indicate that molasses samples from different mills may vary in their reaction to temperature over a range of 10 degrees as reflected by their foam-producing properties. The factor effecting this potential gas production is yet to be determined. However, the observation (Table 5) that molasses which once had been heated produced more gas than duplicate samples which had not been heated is suggestive that the greater the temperature at which the molasses is heated in the factory process, the lower the critical foaming temperature and the greater the foaming on subsequent storage. That this observation is worthy of consideration is evidenced by the fact that certain samples (Table 5, Plant A) with a tendency to excessive foaming produce more carbon dioxide at foaming temperatures when heated for sterilization than unsterilized samples. Also, it should be noted (Table 6) that heat-

TABLE 8

Effect of Temperature on Visible Foaming of Molasses

Time (1940)	Temperature of incubation				
	27°C. (80.6°F.)	37°C. (98.6°F.)	45°C. (113°F.)	65°C. (149°F.)	80°C. (176°F.)
	Foam as per cent of original molasses volume				
PLANT A AT FACTORY PUMP					
June 5— 2:00 P.M.	.....	.....	.....	.....	.....
June 6— 8:30 A.M.	.....	14.0	11.0	133.0	106.0
June 7— 8:00 A.M.	.....	20.0	16.0	163.0	64.0
June 8— 8:00 A.M.	5.0	26.0	26.0	104.0	106.0
June 10— 8:30 A.M.	7.0	32.0	67.0	76.0 [1]	113.0
June 11— 8:00 A.M.	7.0	36.0	65.0	30.0	113.0
June 13	8.0	42.0	69.0	0.0	117.0
June 17	10.0	45.0	84.0	0.0	206.0
June 19	.....	47.0	94.0	.....	.....
June 21	.....	47.0	99.0	.....	.....
PLANT A AT STORAGE TANK					
June 6— 8:30 A.M.	3.3	8.6	17.5	81.0	30.0
June 7— 8:00 A.M.	4.9	13.8	25.0	29.0	23.0
June 8— 8:00 A.M.	9.8	19.0	33.0	52.0	41.0
June 10— 8:30 A.M.	8.2	24.0	44.0	19.0	57.0
June 11— 8:00 A.M.	9.8	26.0	46.0	2.4	70.0
June 13	11.5	30.0	52.0	.....	77.0
June 17	13.1	31.0	74.0	.....	155.0
June 19	.....	34.0	84.0	.....	.....
June 21	.....	35.0	90.0	.....	.....
PLANT B—FIRST SERIES					
May 21— 4:00 P.M.	.....	.....	.....	.....	.....
May 22— 8:00 A.M.	.....	.....	.....	34.0	228.0
May 23— 8:00 A.M.	.....	.....	.....	86.0	228.0
May 24— 8:00 A.M.	.....	.....	.....	86.0	228.0
May 25— 8:00 A.M.	.....	.....	6.0	86.0	228.0
May 27— 8:00 A.M.	.....	.....	24.0	86.0	228.0
May 29— 9:00 A.M.	.....	.....	34.0	194.0	228.0
June 5	.....	.....	64.0	194.0	228.0
June 8	.....	.....	68.0	194.0	228.0
June 11	.....	.....	76.0	194.0	228.0
PLANT B—SECOND SERIES					
June 5—10:00 A.M.	.....	.....	.....	.....	.....
June 6— 8:30 A.M.	1.6	8.5	12.0	7.0	96.0
June 7— 8:00 A.M.	2.0	8.5	15.0	4.0	20.0
June 8— 8:00 A.M.	2.0	7.2	16.0	0.0	0.0
June 10— 8:30 A.M.	2.0	8.5	18.0	0.0	14.0
June 11— 8:00 A.M.	2.0	8.5	20.0	0.0	28.0
June 13	2.0	8.5	26.0	0.0	44.0
June 17	2.0	7.7	40.0	0.0	52.0
June 19	.....	8.5	46.0	.....	.....
June 21	.....	8.5	48.0	.....	.....

[1] In certain cases of excessive foaming the molasses subsides upon explosive escape of gas.

sterilized samples produced appreciably more carbon dioxide when incubated at lower temperatures (1.1°C.) than the unsterilized samples.

To determine further if foaming at lower temperatures is a constant characteristic of certain molasses samples, one part of a sample (Plant A—Storage Tank) with a relatively low critical foaming temperature, was mixed with two parts of molasses (Plant B) with a higher critical foaming temperature and incubated at 65°C. for seven days. This temperature is well above the critical foaming temperature of both samples and the volume of foaming was used as an index of the effect of the samples on each other. It was found (Fig. 3) that molasses which foamed

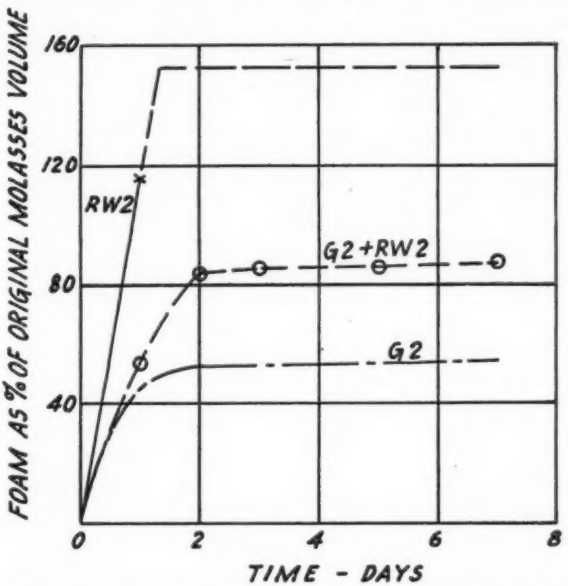

FIG. 3. Effect of mixing molasses of low (RW2) critical foaming temperature with molasses of high (G2) critical foaming temperature on visible foaming when incubated at 65°C.(149°F.).

readily (Plant A, RW2), even when mixed with more stable samples (Plant B, G2), maintained its foaming properties. The inherent foaming factor seemed to be distinct and not affected by dilution with non-foaming molasses. In like manner non-foaming molasses when contaminated with molasses with a lower critical foaming temperature evidenced an increased tendency to foam. This observation may indicate operation of the law of mass action insofar as the amino acids in the foaming molasses are concerned.

A study of a second series (Table 8) of samples from another plant (B) indicated that the amount of foam increased at the higher temperatures and that the critical temperature at which significant foaming may occur is much higher than in any of the Plant A samples studied. This type of molasses consequently could be stored at a higher temperature be-

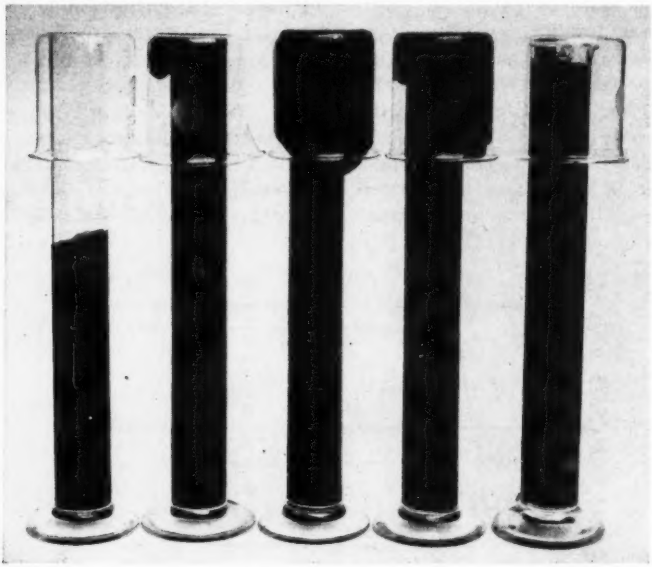

PLATE 1. Comparable volumes of storage molasses from Plants B-C-A-A C, respectively, held at 80°C.(176°F.) for a few hours to indicate relative reaction to high temperatures as evidenced by increase of molasses volume through formation of gas.

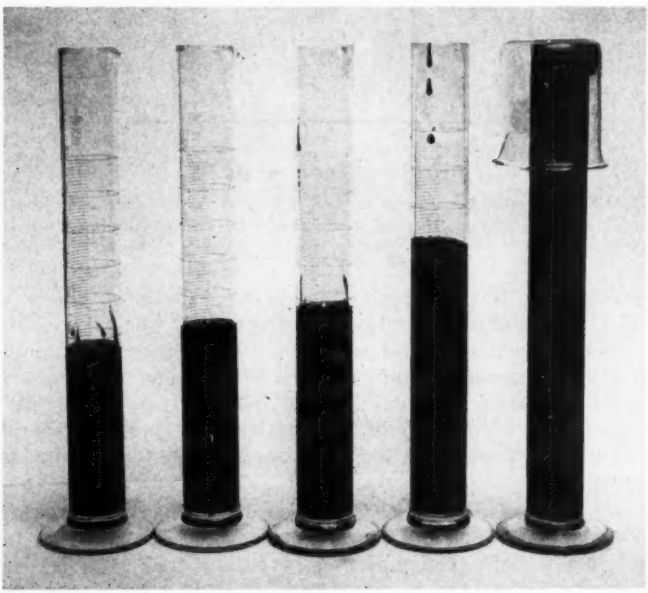

PLATE 2. Comparable volumes of molasses held at 20, 30, 45, 65, and 80°C.(68, 86, 113, 149, and 176°F.) for a few hours, showing effect of temperature on gas production in storage.

fore significant visible foaming occurs. These critical temperatures may vary from season to season depending upon viscosity, specific gravity, and particularly the temperature to which the molasses has been subjected in the factory process.

Effect of Dilution on Foaming: It has been assumed by a number of investigators that the ability of molasses to foam at high temperatures is due indirectly to its specific gravity and more directly to its viscosity. Samples of molasses (Plant A) from the centrifugal (Table 9) were diluted with various amounts of water and subsequently incubated at 65°C.(149°F.) in experimental cylinders. This temperature was selected inasmuch as most samples of molasses will readily foam in a short period when incubated at such a temperature but will not foam owing to the activity of microorganisms.

TABLE 9

Effect of Addition of Water on Visible Foaming of Molasses

Time (1940)	Control—no water added	25 per cent water added
	Foam as per cent of original molasses volume	Foam as per cent of original molasses volume
June 10—3 P.M.	0	0
June 11—8 A.M.	20	3
June 12—9 A.M.	67	3
June 13—8 A.M.	110	25
June 14—8 A.M.	112	20
June 15—4 P.M.	110	25
June 17	108	27
June 19	108	36

TABLE 10

Effect of Water Dilution on Visible Foaming of Molasses

Time (1940)	Foam as per cent of original molasses volume				
	Control—no water added	1 per cent water added	5 per cent water added	10 per cent water added	20 per cent water added
6/12—11 A.M.	0	0	0	0	0
6/13— 8 A.M.	60	70	61	41	0
6/14— 8 A.M.	100	82	15	40	1
6/15— 4 P.M.	50	80	61	10	10
6/17	55	70	63	63	11
6/19	70	70	63	10	15
6/21	40	70	40	60	60

It was found (Tables 9, 10) that a dilution of the molasses materially reduced the amount of foaming. The amount of water necessary to reduce foaming to a minimum, however, was excessive and not practical as a means of controlling foaming in storage tanks. Such data also indicate that the specific gravity of the molasses may be an important factor in molasses foaming particularly at the higher temperatures or, more probably, the dilution of the amino acids.

Effect of Addition of Phenol: If the presence of microorganisms in molasses influenced to any degree the amount of visible foam produced,

the addition of an appreciable quantity of phenol should materially affect foaming when such treated molasses is held at a high temperature. A series of samples was treated with 25 per cent phenol and held in cylinders at 65°C. for eight days. The addition of phenol reduced, but not significantly, the amount of foam; at the end of two days 53 per cent foam was observed.

Relation of Calcium Content of Molasses to Visible Foaming: It has been suggested by many observers that the amount of visible foaming in molasses is in proportion to the calcium content. Earlier observations at two mills indicated that this might be true inasmuch as the calcium oxide content of the foaming molasses was appreciably higher than the non-foaming molasses (Table 11). However, a year later one of these mills (Plant A) showed excessive foaming but also showed a reduced calcium content, indicating that excess lime may not be as important a factor as many have believed.

TABLE 11

Calcium Oxide Content of Final Molasses (Plants A and C)

Sample		Calcium oxide in final molasses			
		1939		1940	
No.	Source	Plant A	Plant C	Plant A	Plant C
		pct.	*pct.*	*pct.*	*pct.*
1	First week of grind	1.80	.....	1.80	1.44
2	Second week of grind	2.02	1.84	1.50	1.30
3	Third week of grind	2.04	2.22	1.54	1.78
4	Fourth week of grind	2.46	2.10	1.68	1.94
5	Fifth week of grind	2.32	2.18	1.86	1.98

CONCLUSIONS

1. The contributing cause of excessive foaming of storage molasses is the temperature at which the molasses is held as it may affect an amino acid and glucose reaction.

2. The critical temperature at which significant foaming occurs varies for different types of molasses. The critical foaming temperature was found at one mill to be approximately 40°C.(104°F.) when molasses was held for more than two weeks. Molasses from a second mill could be held at a slightly higher temperature without excessive foaming, while a third sample could be held at as high as 45°C.(113°F.) for short periods without undue foaming.

3. The calcium oxide content of the molasses, if below two per cent of calcium oxide in the ash, does not appear to be a significant factor.

4. Microorganisms may play a minor part in excessive foaming in certain types of molasses if conditions have allowed the presence and development of gas-producing, heat-resistant types. The greatest activity of these gas-producing types is in the range at which molasses is usually stored, i.e., 25 to 65°C.(77 to 149°F.). Within this temperature range excessive foaming may be due to a combination of the activity of microorganisms and a spontaneous chemical reaction. However, the effect of heat even within these ranges of temperature is of far greater significance than the action of microorganisms.

RELATION OF VARIETY AND STAGE OF DEVELOPMENT TO COMPOSITION OF FIGS

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The production of figs is an important industry in California. Although the cultural aspects have been considered by Condit (1941); there is little information concerning the relation of variety, development of the fruit, or caprification to the composition of figs. Calimyrna figs require caprification (insect pollination) whereas Adriatic, Black Mission, and Kadota varieties are self-pollinated. The latter three, however, may be insect pollinated and when so, frequently show changes in structure and appearance. The Kadota variety shows changes that can be detected easily, and for this reason it was used in the caprification studies.

Condit (1927, 1933) gave analysis for Adriatic figs produced in California as follows: green fruit with eyes fairly well opened, 9.1 per cent sugar and 83.2 per cent moisture; shrivelling fruit with red pulp, 43.3 per cent sugar and 52.4 per cent moisture; dried fruit, 56.81 per cent sugar and 29 per cent moisture.

Colby (1893-94) analyzed 41 samples of California figs, representing 29 varieties, for total solids, proteins, acid, sugar, and ash content. These analyses, however, were only of fully mature figs. Analyses of figs produced in other countries are given by Groce (1928), Argentina; Pellicano (1907) and Siniscalchi 1911), Italy; Azadian (1927) and Sapozhnikova (1940), Caucasia; and Thompson (1914), Hawaii.

Guglielmi (1908) stated that the fat and cellulose content in caprified figs were higher and the sugar content lower than in uncaprified figs. According to data given by Condit (1927), on the other hand, caprification tended to increase reducing sugars as well as crude fiber in Kadota figs but reduced them in the Endish variety. Cruess and Albro (1920) also found that caprified Kadota figs contained more sugar than uncaprified figs.

EXPERIMENTAL PROCEDURE

Samples of Adriatic, Black Mission, Calimyrna, and Kadota varieties of figs were collected at 15-day intervals from certain trees in the same fig-producing district (Figarden, near Fresno, California). Since figs produce two or more crops each year, samples were collected of the first two crops. Five samples were obtained of the first crop and two of the second one. It is more difficult to distinguish between these two crops during the earlier than the later stages of their development. The dates of collection and characteristics of the fruit harvested are given (Table 1). Dried caprified and uncaprified Kadota figs were also obtained from trees in the Fresno area for comparison.

All analyses were conducted in accordance with the procedures of the Association of Official Agricultural Chemists (1935) unless otherwise indi-

cated. For reducing sugars two to four grams of ground dried fruit or 10 grams of ground fresh fruit were used. These samples were mixed with one to two grams of $CaCo_3$ and boiled for 30 minutes with 200 ml. of distilled water. Sugars were determined by use of the Shaffer and Hartman (1921) iodometric method. The Lothrop and Holmes (1931) procedure was employed for glucose, and fructose was determined by difference. Sucrose was determined after inversion with HCL, and starch was determined after treatment with diastase, Woodman (1931). The use of acid hydrolysis gave high values indicating the presence of polysaccharides other than starch, probably pentosans.

TABLE 1

Characteristics of Figs and Data When Harvested

No.	Date harvested	Approximate dimensions of fruits (inches)	Outside color [1]	Stage of maturity
First crop				
1	May 30	0.4 x 0.4	Green	Immature
2	June 15	0.5 x 0.6	Green	Immature
3	June 30	0.8 x 1.3	Yellow green or purplish	Immature
4	July 15	1.3 x 1.6	Amber yellow or dark purple	Firm ripe
5	July 30	1.5 x 2.0	Amber yellow or dark purple	Soft ripe
Second crop				
6	July 25	1.2 x 1.7	Yellow green or purple	Firm ripe
7	August 10	1.7 x 2.2	Amber yellow or black	Soft ripe

[1] The Black Mission figs turn purple or black as they mature, whereas the other varieties turn an amber yellow.

All determinations were made in duplicate and the values given represent an average of the two determinations. To facilitate comparison the data are presented on the moisture-free basis.

DISCUSSION OF RESULTS

Determinations were made of moisture, reducing sugars, glucose, fructose, sucrose, starch, polysaccharides other than starch, crude fiber, alcohol precipitate, pectic acid, protein and ash. The results obtained indicate that reducing sugars increased slowly during the first six weeks of development and rapidly during the last two weeks. Polysaccharides other than starch, crude fiber, pectic substances, protein, and ash, on the other hand, decreased slowly during the early weeks of development and rapidly during the two last weeks. The samples of the second-crop figs obtained showed changes corresponding to those occurring in the first crop of figs during the last two weeks of development. The data obtained are summarized (Tables 2, 3, 4, and 5) and are discussed in the following paragraphs.

Moisture: The moisture content of the fruit remained almost constant during the first six weeks of development but decreased considerably in

the last two weeks (Table 2). The samples of the second-crop fruit harvested in August contained less moisture than those obtained in July. The greatest decrease in moisture occurred during the period of rapid increase of reducing sugars in the fruit.

Reducing Sugars: The reducing sugars of all four varieties increased slowly during the early stages of development and rapidly during the later stages (Tables 3 and 4). The Calimyrna variety contained a lower percentage of reducing sugars than the other varieties studied.

At full maturity the second-crop samples of Calimyrna contained more sugar than those of Black Mission and Kadota but less than the respective first-crop figs of these varieties. Mature first- and second-crop Adriatic figs contained about the same percentage of sugar.

The changes in glucose and fructose content of figs during development followed those of the reducing sugars, as previously indicated. The glucose content of the fruit was greater, in all instances, than that of fructose regardless of variety, crop, or time of harvesting. The ratio of glucose to fructose, however, did not remain constant during the develop-

TABLE 2

Moisture Content[1] of Figs at Various Stages of Development

Variety	First crop harvested					Second crop harvested	
	5-30[2]	6-15	6-30	7-15	7-30	7-25	8-10
Adriatic	85.0	84.4	85.0	82.9	75.0	82.0	70.0
Black Mission	85.0	84.7	84.0	84.3	71.1	83.0	68.0
Calimyrna	82.5	82.0	82.2	82.2	73.6	82.0	72.7
Kadota	82.0	81.7	82.0	83.2	71.8	82.2	71.0

[1] Per cent of total weight of fruit. [2] Date harvested.

ment of the fruit but varied more or less inconsistently in samples collected up to and including July 15. Fruit collected 15 days later showed a definite decrease in the glucose to fructose ratio in all varieties. Second-crop figs harvested in August showed a similar decline (Tables 3 and 4).

Sucrose: Sucrose was present in relatively small quantities in the four varieties studied. The variations in sucrose content observed during development were inconsistent and showed no definite trend.

Starch: When determinations were made by the use of amylase, starch was found to be absent in all samples analyzed. The figures given (Tables 3 and 4) are not considered significant. When determinations were made after acid hydrolysis, on the other hand, relatively high increases were obtained, indicating the presence of polysaccharides other than starch, possibly pentosans. The data obtained by using acid hydrolysis are calculated as polysaccharides other than starch. These acid hydrolyzable polysaccharides were abundant in the most immature figs and decreased to a relatively small percentage as the fruit matured.

Crude Fiber: Varieties containing many seeds or woody flesh would be expected to contain relatively high percentages of crude fiber. In view of the presence of many large seeds in Calimyrna figs they might be expected to fall into this category. The data (Tables 3 and 4), however,

TABLE 3

Analyses[1] of Adriatic and Calimyrna Figs at Various Stages of Maturity[2]

Constituent	Adriatic							Calimyrna						
	First crop harvested					Second crop harvested		First crop harvested					Second crop harvested	
	5-30	6-15	6-30	7-15	7-30	7-25	8-10	5-30	6-15	6-30	7-15	7-30	7-25	8-30
Reducing sugars	22.00	25.00	27.30	28.70	76.40	23.60	76.00	15.50	18.30	20.20	22.20	60.60	22.16	64.40
Glucose	16.00	17.80	19.80	20.50	45.00	20.50	46.00	11.45	13.20	14.80	15.70	34.10	16.20	40.18
Fructose	6.60	7.69	8.00	8.77	33.70	8.60	32.10	4.30	5.55	5.80	6.80	26.80	6.00	26.20
Ratio $\frac{\text{glucose}}{\text{fructose}}$	2.40	2.30	2.47	2.34	1.36	2.38	1.40	2.60	2.40	2.55	2.30	1.27	2.70	1.53
Sucrose	0.13	1.20	1.00	1.17	2.40	0.68	1.30	1.02	1.66	2.80	3.30	4.10	1.70	0
Starch	.....	.....	.....	0.60	0.36	0.90	0	.....	.....	.....	.....	0	0.55	2.20
Polysaccharides other than starch	19.20	11.50	5.60	4.60	2.50	3.90	1.00	18.80	18.80	16.20	6.60	.....	6.30	3.90
Crude fiber	15.30	12.82	12.00	9.35	6.60	8.30	3.80	7.42	7.10	7.00	6.33	3.78	6.44	3.95
Alcohol precipitate	10.66	9.70	12.00	9.80	3.28	7.70	3.06	10.28	9.40	11.23	8.88	2.60	8.44	2.50
Pectic acid	7.80	6.73	6.25	5.85	1.24	4.70	2.20	7.14	6.50	5.80	5.00	1.20	.....	.....
Protein	15.30	12.90	12.00	9.80	4.25	9.66	4.06	14.28	12.22	11.23	10.40	4.24	10.66	4.60
Ash	8.00	7.60	7.90	5.60	3.20	6.70	3.60	9.70	8.00	7.00	4.70	3.10	5.00	4.40

[1] Given in percentages on moisture-free basis. [2] Characteristics of figs picked on different dates are given in Table 1.

TABLE 4

Analyses[1] of Kadota and Black Mission Figs at Various Stages of Maturity

Constituent	Kadota							Black Mission						
	First crop harvested					Second crop harvested		First crop harvested					Second crop harvested	
	5-30	6-15	6-30	7-15	7-30	7-25	8-10	5-30	6-15	6-30	7-15	7-30	7-25	8-10
Reducing sugars	16.55	16.40	20.00	25.00	75.00	21.30	71.70	16.40	18.70	20.00	24.20	79.50	30.00	74.53
Glucose	12.20	11.80	14.70	19.60	43.60	15.95	42.00	11.00	12.90	13.80	16.56	46.70	23.00	47.23
Fructose	4.83	5.00	5.66	8.33	37.20	6.28	30.90	5.70	6.10	6.60	8.28	35.50	7.80	29.50
Ratio $\frac{\text{glucose}}{\text{fructose}}$	2.50	2.36	2.50	2.35	1.17	2.53	1.30	1.93	2.10	2.00	2.00	1.31	2.90	1.60
Sucrose	0.33	1.63	1.10	1.60	3.97	0.84	0.07	0.13	0.90	1.25	0.95	2.70	0.53	2.60
Starch	.....	.....	.....	.....	0.24	0	0.34	.....	.....	.....	0.30	0.48	0	0
Polysaccharides other than starch	21.40	23.40	21.10	11.20	2.90	11.10	.....	28.00	24.80	21.20	16.50	2.40	10.00	1.25
Crude fiber	13.00	11.96	11.10	10.30	3.80	10.90	4.10	10.00	9.15	8.00	7.64	3.60	7.60	3.65
Alcohol precipitate	11.66	10.92	13.33	10.40	2.62	9.40	2.80	13.20	12.09	14.69	11.46	2.96	8.35	2.60
Pectic acid	5.90	5.60	4.94	4.50	1.73	4.16	1.30	8.00	7.50	6.87	6.03	1.73	5.80	1.30
Protein	13.00	11.96	11.10	10.80	3.90	11.40	4.24	14.73	12.28	11.25	9.80	3.66	11.20	4.78
Ash	7.70	7.00	6.60	5.40	3.30	5.40	3.40	9.00	8.50	8.10	7.20	3.10	7.60	3.70

[1] Given in percentages on moisture-free basis.

show that the Calimyrna samples contained comparatively low percentages of crude fiber, whereas the Adriatic figs were comparatively rich in crude fiber. In all instances crude fiber decreased gradually during development of the fruit. Mature first and second crop figs contained about the same quantities of crude fiber.

Alcohol Precipitable Matter and Pectic Acid: The changes observed in alcohol precipitable matter of the fruit were gradual and consistent as was the case also for pectic acid. During the late stages of development, however, all varieties showed a rapid decrease in alcohol precipitable matter. Pectic acid, on the other hand, gradually decreased until late in the stage of development of the fruit when the decreases were rapid (Tables 3 and 4). Changes in alcohol precipitable matter and pectic acid were not parallel throughout the development of the fruit. These substances form an important part of the immature fruit but are relatively

TABLE 5

Composition of Caprified and Uncaprified Kadota Figs (Moisture-Free Basis)

Constituent	Caprified	Caprified	Uncaprified
	pct.	*pct.*	*pct.*
Reducing sugars	64.90	66.00	60.00
Glucose	42.00	43.60	39.95
Fructose	24.60	24.20	22.10
Sucrose	0.50	0.10	0.60
Starch	0.00	0.00	0.20
Crude fiber	3.00	2.90	3.90
Protein	3.60	3.80	2.20
Ash	5.32	4.87	2.42

unimportant as constituents of the mature fruit. Alcohol precipitable matter decreased according to variety in the following order: Black Mission, Kadota, Adriatic, and Calimyrna. The pectic acid content decreased according to variety as follows: Black Mission, Adriatic, Calimyrna, and Kadota. Mature first-crop and second-crop samples of fruits, irrespective of variety, were similar with respect to alcohol precipitable matter and pectic acid.

Protein: The changes in nitrogen content of figs during development were similar to those for pectic acid, a slow decrease at first and a rapid decrease during the final stage of development. The protein content of the different varieties and crops analyzed were quite similar where corresponding samples were compared (Tables 3 and 4).

Ash: The course of change in ash content during development and maturation is quite similar to that of the protein content. Small, immature figs contain a surprising quantity of ash which apparently decreases as the fruit matures because of dilution resulting from the great increase in sugar.

Effect of Caprification: Kadota figs produced in orchards located near Calimyrna orchards in which caprification is practiced, frequently become caprified. Kadota figs that have been caprified can be differentiated from uncaprified Kadota figs by difference in size, color, and internal structure.

The caprified figs are usually larger, lighter in skin color, and contain larger seeds. Growers also maintain that there is a difference in internal color but this is not obvious to the average individual. There is a diversity of opinion concerning the effect of caprification on the composition of Kadota figs. The literature on this controversy has already been reviewed. The results of analyses of caprified and uncaprified Kadota figs from our tests are given (Table 5). Caprified figs contained higher percentages of reducing sugars, ash, and protein but a slightly lower percentage of crude fiber. The observations on sugar content are in accordance with those of Cruess and Albro (1920) but disagree with those of Guglielmi (1908).

SUMMARY AND CONCLUSION

Adriatic, Black Mission, Calimyrna, and Kadota figs collected during their development at 15-day intervals increased gradually in sugar content during the first six weeks and rapidly during the last two weeks. Polysaccharides other than starch, crude fiber, alcohol precipitable matter, pectic acid, protein, and ash, on the other hand, decreased slowly during the first six weeks and rapidly in the last two weeks of development. The great increase in sugar content that occurred was accompanied by softening and coloration of the fruit. The decrease in ash and possibly other constituents can be explained, in part at least, by dilution resulting from the increase in sugar content.

Starch and sucrose were found to be present in small quantities or entirely absent.

Varietal differences in composition are summarized in tables. In contrast to common belief Calimyrna figs were lowest in sugar content.

Fully matured samples of first- and second-crop figs were quite similar in composition. Second-crop figs collected during the later stage of development showed changes similar to those in corresponding samples of first-crop figs.

Caprified Kadota figs were found to contain more sugar than uncaprified figs from the same orchard.

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EFFECT OF FREEZING AND OF CANNING IN GLASS AND IN TIN ON AVAILABLE IRON CONTENT OF FOODS[1,2]

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The studies made to date on the availability of iron in foods have been concerned chiefly with fresh foods. Since much of the food consumed today has been preserved in some way, it is desirable to know whether preservation has any effect on the availability of iron in such food. A preliminary study by Hastings, Fellers, and Fitzgerald (1941) indicated that the commercial quick-freezing of fruits, vegetables, poultry, and fish increased the availability of the iron present. In the present study, the effects of canning in glass and tin and of storage in tin cans on iron availability were determined. The study of the effects of freezing were also continued. The foods employed were peaches, asparagus, sweet corn, green snap beans, spinach, lima beans, and red sea perch fillets.

PREVIOUS REPORTS

There is very little reported in the literature on the effects of canning on the availability of iron in foods. The study by Hastings, Fellers, and Fitzgerald (1941) on the effects of freezing on iron availability has been mentioned above. Several investigators, such as Elvehjem, Hart, and Sherman (1933); Sherman, Elvehjem, and Hart (1934); Shackleton and McCance (1936); Smith and Otis (1937a,b); Ascham, Speirs, and Maddox (1938); Yang and Dju (1939); and McCance and Widdowson (1940), have reported data on the total and available iron content of fresh foods and, to a lesser extent, cooked foods.

SAMPLE PREPARATION

The frozen foods and a portion of the fresh foods were dried as received. The remainder of the fresh foods were canned in glass jars of the "lightning" type, and in C-enamel (zinc-enamel), fruit enamel, or plain tin cans as indicated (Table 1). The glass jars were opened and the contents dried after about one month. The tin cans were opened at 30, 100, and 200 days after canning, and the contents dried.

With the exception of the peaches dried after blanching, peaches were mixed with iron-free starch before drying, in order to facilitate drying.

The food materials were dried on wax paper or in aluminum dishes in a tunnel dryer at 48.9°C.(120°F.). The materials were then put through a newly galvanized food chopper, and drying was continued until the

[1] This report represents the continuation of a project originated by W. H. Hastings. Acknowledgment is also due V. F. Coutu, who did the canning and drying of several of the materials at the start of the project.

[2] Contribution No. 438, Massachusetts Agricultural Experiment Station, Amherst, Mass.

TABLE 1

Availability of Iron by Chemical and Rat Bioassay Methods (Dry Basis)

Food	pH value	Total iron	Available iron	Proportion of total iron as available iron	
				Dipyridyl method	Bioassay method
		p.p.m.	*p.p.m.*	*pct.*	*pct.*
Sweet corn (fresh)	6.7	21	20	95	....
Sweet corn (quick-frozen)	....	19	19	100	....
Sweet corn (glass-canned)	6.6	21	21	100	....
Sweet corn (C-enamel cans)					
After 30 days	6.5	26	24	92	....
After 100 days	6.5	27	26	96	....
After 200 days	6.5	27	26	96	....
Asparagus (fresh)	5.8	96	59	61	....
Asparagus (quick-frozen)	6.2	73	44	60	55
Asparagus (slow-frozen)	6.5	72	45	63	....
Asparagus (glass-canned)	5.4	87	49	56	....
Asparagus (plain tin)					
After 30 days	5.3	108	55	51	....
After 100 days	5.3	106	52	49	....
After 200 days	5.2	96	47	49	....
Green snap beans (fresh, blanched)	....	130	47	36	....
Green snap beans (quick-frozen)	6.2	150	91	61	59
Green snap beans (glass-canned)	5.5	107	45	42	....
Green snap beans (plain tin)					
After 30 days	5.5	189	81	43	....
After 100 days	5.4	207	96	46	....
After 200 days	5.4	212	102	48	....
Lima beans (fresh, blanched)	....	68	46	68	....
Lima beans (quick-frozen)	6.5	85	62	73	70
Lima beans (glass-canned)	6.1	62	43	69	....
Lima beans (plain tin)					
After 30 days	5.9	73	45	62	....
After 100 days	6.0	92	58	63	....
After 200 days	5.9	97	63	65	....
Lima beans (C-enamel)					
After 30 days	6.0	71	44	62	....
After 100 days	6.0	75	47	63	....
After 200 days	6.0	78	49	63	....
Spinach (fresh)	6.4	350	84	24	....
Spinach (quick-frozen)	6.7	190	120	63	42
Spinach (glass-canned)	6.0	560	253	45	....
Spinach (plain tin)					
After 30 days	6.0	545	287	52	....
After 100 days	6.0	570	302	53	....
After 200 days	5.9	590	320	54	....
Red sea perch (fresh)	6.8	46	9	20	....
Red sea perch (quick-frozen)	6.8	42	8	19	17
Red sea perch (glass-canned)	6.9	49	4	8	....
Red sea perch (C-enamel)					
After 30 days	6.9	36	4	11	....
After 100 days	6.9	25	2	8	....
After 200 days	6.9	24	2	8	....
Peaches (freestone) in 50% syrup					
Dried after blanching	3.9	92	....[1]	....	....
Glass-canned	4.0	180	40	22	....

TABLE 1 *(Concluded)*

Food	pH value	Total iron	Available iron	Proportion of total iron as available iron	
				Dipyridyl method	Bioassay method
Fruit enamel cans		*p.p.m.*	*p.p.m.*	*pct.*	*pct.*
After 30 days	3.9	194	44	23	....
After 100 days	3.9	205	52	25	....
After 200 days	3.9	209	55	26	....
Plain tin cans					
After 30 days	3.9	234	56	24	....
After 100 days	3.9	239	57	24	....
After 200 days	3.9	288	77	27	....
Peaches (freestone) water pack					
Dried after blanching	3.6	78	....[1]	....	....
Glass-canned	....	119	25	21	....
Fruit enamel cans					
After 30 days	3.8	127	25	20	....
After 100 days	3.6	135	34	25	....
After 200 days	3.7	140	38	27	....

[1] Iron-dipyridyl color obliterated by natural red color of the extract. This difficulty was not encountered in the peaches dried with starch.

weight was constant. The materials were finally pulverized in a ball mill. Shackleton and McCance (1936) recommend using the undried material as consumed, but this procedure makes accurate sampling difficult and checking by rat bioassay impossible.

CHEMICAL METHODS FOR AVAILABLE IRON

The chemical test for available or "ionizable" iron originally proposed by Hill (1930), based on the formation of a colored complex of ferrous iron with alpha alpha-dipyridyl, has had nearly as many modifications as it has had users. Discrepancies have appeared in the values of iron availabilities reported and have been attributed often to differences in the methods of analysis. An equally tenable explanation is that the availability of iron in any given product may vary widely with variety, conditions of growth, maturity, etc. Holland and Ritchie (1941) have reported wide variations in total iron content of certain food materials; for example, the iron content of spinach ranged from 384 to 1,258 p.p.m., celery from 53 to 484 p.p.m., corn from 16 to 53 p.p.m. No comparative studies have been made for available iron, but similar variations could be expected.

TOTAL AND AVAILABLE IRON DETERMINATIONS

Total iron was determined by the A.O.A.C. (1940) thiocyanate method. The ferric thiocyanate colors were measured in the Evelyn colorimeter, with Filter 490 millimicrons, and the amounts of iron were read from a standard curve obtained by treating a series of ferric chloride solutions containing from 0 to 100 gamma of iron by the same method.

The method of determining available iron employed was that of Hill (1930), as modified by Elvehjem, Hart, and Sherman (1933) and by Shackleton and McCance (1936). The procedure which was adopted after a preliminary study of alternative methods, was as follows: .5 to 2.0

grams of finely ground, dried material were weighed into tubes graduated at 50 ml.; 10 ml. of sodium acetate-acetic acid buffer of pH 5.5, 10 ml. of distilled water, and 1.0 gram of sodium hydrosulphite were added (.25 gram of hydroquinone may be used instead of the hydrosulphite if the material is not pigmented). The contents of the tubes were well mixed with a glass rod and allowed to stand overnight with occasional stirring. The volume was then made up to 50 ml. and the contents were again well mixed. The tube was stoppered and allowed to stand overnight with occasional stirring. The contents were then shaken thoroughly and filtered through ashless filter paper (Whatman No. 41). The first 30 ml. were caught and the remainder discarded. The solutions were divided into two approximately equal portions. A few small crystals of alpha alpha-dipyridyl were added to one portion; and the other portion was used as the blank. The color was measured in an Evelyn photoelectric colorimeter, with Filter 520 millimicrons, using a nulled blank. This type of instrument allows the analysis of turbid and slightly colored extracts without significant error. The amount of iron present in each solution was then read from a standard curve obtained by treating a series of ferric chloride solutions containing from 0 to 100 gamma of iron by the method described above.

The amount or iron added by the reagents was determined for each series of determinations, and corrections were made. The amount was usually nil, and never exceeded three gamma.

RAT BIOASSAY FOR IRON AVAILABILITY

The biological method of testing iron availability involves the use of rats depleted of their iron to a low level of hemoglobin. The amount of hemoglobin regenerated in rats fed the test foods for a given length of time is compared with the hemoglobin regeneration in rats fed a whole-milk diet to which a known amount of iron, 100 per cent available, is added as a salt.

The samples of dried, frozen foods were the only samples of which there was sufficient quantity to provide for a six week's bioassay. Hence only a few bioassays were run. However, these serve to check the accuracy of the chemical determinations of available iron, as the chemical determinations on the other materials can be compared with those on the frozen materials.

The technique employed was that of Elvehjem and Kemmerer (1931) with the incorporation of suggestions by Smith and Otis (1937a,b) that the amount of available iron fed daily should be .15 mg. or less, and that .05 mg. of copper as the sulphate and .04 mg. of manganese as the chloride be added to the diet when the animals are 28 days old.

The rats were weaned at 21 days and placed on a powdered-milk diet until the hemoglobin level was about four grams per 100 c.c. of blood. This level was reached when the rats were 60 to 70 days old, and 90 to 110 grams in weight. Weight and hemoglobin were recorded weekly throughout the assay. Six rats were used for each supplement and for the negative and positive control groups. The standard iron controls received the basic milk ration plus iron from ferric chloride at the level of .15 mg. of iron

daily. The dried-food supplements were fed daily in amounts calculated to contain .15 mg. of available iron as determined by the dipyridyl method, with the exception of the red sea perch, which had to be fed on the basis of total iron because of the low content of available iron.

The hemoglobin was measured by the Newcomber method, i.e., by comparing calibrated dilutions of acid hematin with a Bausch and Lomb standard in a Klett colorimeter. Blood was obtained from the rat's tail with a blood pipette graduated into .01 and .02 c.c. Dilutions were made in one-tenth normal hydrochloric acid, and allowed to stand one hour before measuring.

In a six weeks' assay period, the hemoglobin regeneration is directly proportional to the amount of available iron fed, up to a level of .15 mg. of iron daily, Smith and Otis (1937a). The amount of available iron in the daily food supplement was therefore calculated from the following:

$$\frac{\text{hemoglobin increase on food supplement} \times 0.15 \times 1000}{\text{hemoglobin increase on ferric chloride} \times \text{daily weight of food supplement}}$$

which gave the available iron content of the supplement in parts per million.

DISCUSSION OF RESULTS

The results of the rat bioassays (Table 1) checked fairly well with the chemical determinations of available iron, with the exception of the spinach determinations. This vegetable had a strong cathartic action on the rats, so that the discrepancy in results in this case may be ascribed to a too rapid passage of the material through the intestinal tract as well as to a failure of the chemical method.

The total iron content of the frozen foods was generally lower than that of the fresh. This may be attributed to the loss of soil particles in the more thorough washing and blanching operations to which the vegetables were subjected before commercial quick-freezing.

The percentage availability of iron was generally higher for the frozen foods as compared with either the fresh or the canned foods. Hastings, Fellers, and Fitzgerald (1941) have suggested that this may be the result of a disintegration of the tissues and protoplasm by the freezing process itself.

The total and available iron in the glass-canned materials were about the same as in the fresh in most cases. The greater amount of total iron in the glass-canned spinach may have been due to a less thorough washing of that particular sample. It may be said that canning in glass has no adverse effect on availability of iron.

The observed changes in total and available iron in foods canned in tin are correlated somewhat with the hydrogen-ion concentrations of the materials. Peaches (pH about 3.9) gained considerable iron in plain tin cans, but less in fruit enamel. Less acid foods gained less iron; asparagus (pH about 5.4) lost a very slight amount, and could be said to be unchanged; while the red sea perch (pH 6.9) lost 52 per cent of its total iron and virtually all of its available iron in 200 days' storage.

The loss of iron from the fish may be explained on the basis of the reversal in E.M.F. of the iron-tin couple at the pH and ionic concentra-

tions obtained in the cans. This reversal occurs at very low concentrations of ionized tin according to the equation:

$$E = E_{Fe, Fe^{++}} - E_{Sn, Sn^{++}} - \log \frac{(Fe^{++})}{(Sn^{++})}.$$

The greatest changes in iron content took place in the first month of storage. Increases and decreases in total iron were closely paralleled by like changes in available iron. It is evidently largely ionizable or available iron which takes part in these changes.

SUMMARY

The effects of freezing and of canning in glass and in tin cans on total and available iron in foods were studied.

The chemical method for available iron gave consistent results for fruits, vegetables, and fish and checked with the bioassay method.

Commercial quick-freezing of foods was found to increase the availability of iron slightly.

Canning in glass had little or no effect on total and available iron in foods.

Foods canned in tin showed changes in iron content somewhat correlated with their hydrogen-ion concentrations. No change or slight gains in iron were observed with the vegetables, and considerable gains in iron were observed with the more acid product, peaches. Red sea perch, with a pH of 6.9, lost half of its total iron and nearly all of its available iron when packed in tin cans lined with zinc enamel (C-enamel).

Iron gained from the cans seemed to be nearly 100 per cent available.

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BEHAVIOR OF MICROORGANISMS IN FRUIT JUICES AND IN FRUIT JUICE-SUCROSE SOLUTIONS STORED AT —17.8°C.(0°F.)[1]

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Since sucrose is being used in the freezing preservation of fruits and fruit products, it is important to establish its relation to the destruction or survival of microorganisms which become incorporated in the packs during processing. Not only should contaminating microorganisms be kept at a minimum by the employment of sanitary packing methods, but every precaution consistent with the maintenance of a good-quality product should be taken to hasten microbial destruction during freezing storage. A low microbial count will help insure favorable competition for the frozen product, especially if rigid microbiological standards are adopted by the industry. Moreover, a low-count product possesses a potentially longer life than a high-count product when subjected to prevailing storage, transportation, and marketing treatments.

One of the early arguments advanced for the use of sucrose in the preservation of fruits by freezing was that it delayed fermentation and that the fruit could therefore be frozen before any marked deterioration occurred. The continued use of sucrose in the light of modern refrigeration technic has been motivated not so much by its need to delay fermentation as by its ability to aid in preserving desirable characteristics of the fresh fruit. Unfortunately there is reason to believe that sucrose also aids in preserving microbial viability. In fact Keith (1913) kept bacterial cultures covered with 10-per cent sucrose solution in a vigorous condition for eight months at —10°C.(14°F.). "Osmophilic" microorganisms, that is, types capable of growing or reproducing or bringing about fermentation in highly concentrated sucrose solutions at ordinary temperatures, are not the primary spoilage agents surviving in frozen, sucrose-packed fruits. The spoilage microorganisms which do survive are probably less restricted in their requirements. They are composed for the most part of a miscellany of species which are on the fruit at the time of harvesting; some escape rinsing and increase in number whenever there is a delay in processing or when unsanitary processing methods are used. Dry sucrose or a highly concentrated sucrose sirup may kill some of these microorganisms on contact. On the other hand, the sucrose may only reduce life processes or at most exert a bacteriostatic effect. The "preserved alive" microorganisms become active whenever there is a favorable change in their environment, and such a favorable change usually arises when the suspending medium is diluted. Thus conditions become favorable for growth in sucrose packs prior to freezing and again after thawing. Prior to freezing the added sucrose, dry or sirup, is diluted by the water ad-

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hering to the fruit and by the juice extracted from th[illegible]smosis. Both adhering water and sucrose dilute the extracted fru[illegible]. The environment is continually changing but it is changing in a [illegible] less uniform manner throughout the mass in the container. Any [illegible] to an equilibrium simply means too much delay in processing. Free[illegible]nd gravitational forces not only alter the relative proportions of [illegible], water, tissue, and soluble fruit solids in different parts of the same [illegible] but they may also be responsible for changes in the physical state of the same substances. As ice forms the soluble solids become concentrated. At —17.8°C.(0°F.) there may exist in the mass quantities of unfrozen, highly concentrated, fruit juice-sucrose sirup. The extent of the freezing and gravitational redistribution of constituents and the extent of their physical alteration depend upon such factors as kind of fruit or juice, type of pack, freezing and storage temperatures, method and rate of freezing, and nature and size of the container. When the mass is thawed, moisture is made available which may dilute the sucrose and permit growth of microorganisms. The sucrose concentration is low in some sections of the thawed mass but we may reasonably suspect that it may be high enough to offset any germicidal tendencies exhibited by the fruit acids. Microbial growth is likely to occur rapidly in these sections if the temperature is favorable. Furthermore as the concentrated sirup in the core and bottom of the mass gradually becomes diluted the viable microorganisms contained in it find themselves in a medium suitable for growth.

REVIEW OF LITERATURE

The literature treating the freezing preservation of fruits and fruit products is in a large measure concerned with the use of sucrose in frozen whole, sliced, or pulped fruits. The advantages of using sucrose in dry or in sirup form have been listed by Diehl and co-workers (1930) and by Joslyn (1930) and have been recently summarized by French (1940).

When storage conditions have been identical it has been the author's personal observation that microbial destruction is greater in unsweetened than in sweetened frozen berries and berry juices, and greater in sirup-packed than in dry-packed. Undoubtedly other investigators have made similar observations but they have neglected, for the most part, to incorporate such data in their publications. Diehl and co-workers (1930) attributed the greater tendency for the fruit mass in the top and at the center of a barrel to undergo fermentation and spoilage, to the difficulty of retaining sufficient sugar at these points during packing and to difficulties in cooling the fruit. Fellers (1932) examined some fruits which had been frozen in sealed tin cans at —23.3°C.(—10°F.) and which had been held approximately 12 months at —12.2°C.(10°F.). According to his observations, "Fruits packed with sugar had fewest organisms of all," and "Upon thawing, the greatest increase occurred in fruit frozen without sugar." Wallace and Tanner (1934) noticed a slightly greater microbial reduction in sirup-packed than in sugar-packed frozen fruits.

Freezing in still air is used, but less extensively than in former years. Some of the other freezing methods, though slightly more violent in mechanical agitation of the mass, and quicker to bring about freezing, do

not greatly affect the final physical nature of the frozen mass insofar as distribution of the solid and soluble substances is concerned. The frozen mass as prepared for low-temperature storage is not a homogenous mass and it is likely to become even less so as the storage time lengthens. This is significant since any incorporated viable microorganisms are likely to find one or more environmental conditions especially suitable for survival at the low storage temperature. Although it has long been known that freezing and gravitational forces tend to bring about a concentration of any particles suspended in a fluid medium, it is only recently that attention has been given to the idea that the nature of the distribution of solids and soluble substances in the frozen mass may bear some relationship to the quality of a frozen fruit product. Now more attention is being given to the factors which influence distribution and to the manner in which they exert their influence on distribution.

According to tabular data given by Diehl and co-workers for two-plus-one (two parts by weight of berries plus one part by weight of sugar) commercial packs of Marshall strawberries held at —9.4°C.(15°F.), the sirup concentrations in the tops of the barrels ranged from 26 to 44 per cent and in the bottoms, from 44 to 58 per cent. Woodroof (1931) examined samples from different positions in a one-pound package of slow-frozen grape juice and found "a variance from 16 per cent sugar on the side to 62 per cent at the top and 57.7 per cent at the bottom." Berry (1933) found a larger number of viable organisms present per cubic centimeter of concentrated sugar solution in the bottom of containers of small fruits stored at —10°C.(14°F.) than in the freshly thawed, relatively sugar-free ice from the top. Data from McFarlane's (1940) sectional studies, which were made on frozen masses of 10-per cent sucrose solution, cider and sirup-packed raspberries, confirm the observations made by Diehl and co-workers, by Woodroof, and by Berry. Another study by McFarlane (1941), concerned with the influence of sucrose and hydrogen-ion concentration on the behavior of microorganisms at subfreezing temperatures, yielded data which augment those obtained from the sectional studies. The collected data indicate that any orderly relation between sucrose concentration and microbial survival depends upon such factors as nature of the microorganisms, hydrogen-ion concentration of the medium, total acidity of the medium, and storage temperature.

A number of workers have investigated microbial behavior in frozen apple, grape, orange, and other fruit juices and have noted the influence of acidity, temperature, soluble-solids content, etc., on the retention of viability. Among these are Irish and Joslyn (1929), Tanner and Wallace (1931), Beard and Cleary (1932), Wallace and Park (1933), Berry and Diehl (1934), Shrader and Johnson (1934), and McFarlane (1940).

EXPERIMENTAL PROCEDURE

To study the effect of the high sucrose concentrations found in the juice-sucrose-water mixtures in typical fruit packs, experiments were set up in which microorganisms were suspended in a fruit juice and in batches of the same fruit juice containing different concentrations of sucrose. Juice rather than whole fruit was used in order to make possible more uniform

sampling and to eliminate any influence which fruit tissue might have on microbial behavior. Fresh orange juice was prepared in the laboratory. Loganberry juice, a commercially frozen product, was purchased in the open market. Both juices were supercentrifuged to remove the suspended solids. Juice-sucrose solutions were prepared by adding high-quality, commercial cane sugar to batches of juice to give total-soluble-solids contents (as determined by refractometer readings) of 20, 30, 40, 50, and 60 per cent. In Experiment 6 citric acid was added to each loganberry juice-sucrose solution to give a total acidity comparable with that of the original juice. Data are given relative to the soluble-solids contents, hydrogen-ion concentrations, and total acidities of the suspending media (Table 1).

TABLE 1

Soluble-Solids Contents, Hydrogen-Ion Concentrations, and Total Acidities of Juice and Juice-Sucrose Solutions

Medium	Orange juice—Expt. 1, 2, 3			Loganberry juice—Expt. 4, 5, 6					
	Unacidified			Unacidified			Acidified sucrose solutions		
	Solids[1]	pH	Acid[2]	Solids	pH	Acid	Solids	pH	Acid
	pct.			*pct.*			*pct.*		
Juice	10.3	3.6	.91	11.8	3.0	2.04	11.8	3.00	2.04
J + suc. to 20%[3]	20.1	3.6	.84	20.0	3.0	1.89	20.0	2.94	2.09
J + suc. to 30%	30.0	3.6	.76	30.0	3.0	1.70	30.4	2.86	2.02
J + suc. to 40%	40.2	3.6	.69	40.0	3.0	1.51	40.4	2.82	2.05
J + suc. to 50%	50.0	3.6	.62	50.0	3.0	1.32	50.4	2.72	2.06
J + suc. to 60%	.....	....	....	60.0	3.0	1.13	60.2	2.69	2.05

[1] Approximate percentage soluble solids as determined by refractometer readings. [2] Acid = total acid content expressed as grams of citric acid per 100 milliliters of juice. [3] The expression "J + suc. to 20%" means that sucrose has been added to juice in a quantity sufficient to give a total soluble-solids content of 20 per cent.

The microorganisms used were *Escherichia coli*, spores of *Bacillus subtilis*, a pink Torula species isolated from commercially frozen orange juice, *Saccharomyces ellipsoideus* Hansen, and a strain of *Saccharomyces cerevisiae* isolated from a beverage by Hall, James, and Stuart (1933). *B. subtilis* cultures were incubated 38 days; the others, three days at 30°C.(86°F.). The microorganisms were rinsed from the slants with small quantities of sterile water and the resulting heavy "stock" suspensions shaken with sterile glass beads. The vegetative cells of *B. subtilis* were destroyed by heating the suspension in a water bath for 15 minutes at 80°C.(176°F.). Suitable portions of the "stock" suspensions were used in inoculating the juice and juice-sucrose solutions.

Suspensions of *E. coli*, *B. subtilis* spores, and Torula were made in orange juice and in orange juice-sucrose solutions. Similarly suspensions of Torula, *S. cerevisiae*, and *S. ellipsoideus* were made in loganberry juice-sucrose solutions. The well-mixed suspensions were dispensed in 10-ml. quantities in sterile pyrex tubes (20 x 150 mm.); the tubes were stoppered with scalded corks, placed in racks, and frozen and stored in still air at —17.8°C.(0°F.). Samples of each suspension were plated immediately prior to freezing. Duplicate (and in many instances triplicate) stored samples were plated after two and four days and after 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, and 24 weeks, thereafter less regularly unless it seemed

advisable to discontinue altogether the plating of a particular experimental series of samples. One per cent-dextrose agar (pH 6.6-7.0) was used for plating the *E. coli*, *B. subtilis* spores, and Torula in Experiments 1, 2, and 3. Wort agar (pH 5.0) was used for plating the Torula and Saccharomyces species in Experiments 4, 5, and 6. All plates were incubated at 30°C.; *B. subtilis* for two days; *E. coli*, *S. ellipsoideus*, and *S. cerevisiae* for three days; and the Torula species for five days.

EXPERIMENTAL RESULTS

Experiment 1, Table 2—Behavior of E. coli in Orange Juice and Orange Juice-Sucrose Solutions: The suspensions were originally heavy (3.8 to 8.4 million per ml.). Therefore it was not surprising that although large numbers of cells were still viable after 26 weeks of storage in 40-per cent and 50-per cent juice-sucrose solutions, the number surviving in each medium was less than one per cent of the number in the original inoculum. In these two juice-sucrose solutions 99 per cent of the cells had not been destroyed after 20 weeks, whereas in the unsweetened orange juice 99 per cent had been destroyed within 48 hours. Moreover, it was even uncertain whether the samples of unsweetened juice examined after the first week and through the sixth week owed their small but positive counts to viable cells surviving freezing storage or to contamination after removal from storage. Beard and Cleary (1932) and Shrader and Johnson (1934) reported the relatively rapid destruction of some bacterial forms in unsweetened orange juice.

Experiment 2—Behavior of B. subtilis Spores in Orange Juice and Orange Juice-Sucrose Solutions: From one to 1.7 million spores were suspended in the media. After 78 weeks of storage at —17.8°C.(0°F.) the media did not show any marked change in viable spore content regardless of whether they were unsweetened or contained a high concentration of sucrose.

Experiment 3, Table 3—Behavior of a Torula Species in Orange Juice and Orange Juice-Sucrose Solutions: At the termination of 72 weeks of storage there were viable cells in all of the media, although 99 per cent of the cells were destroyed in the unsweetened juice between the 36th and 40th weeks. The viable-cell counts in the juice-sucrose solutions tended to vary directly with the sucrose concentrations and this relationship became more obvious as the storage period lengthened. Less than .1 per cent of the cells in the original inoculum survived 72 weeks in the unsweetened juice. The percentages of cells surviving in the juice-sucrose solutions (given in order of increasing sucrose concentrations of the solutions) were .2, 1.2, 7.2, and 53.9.

Experiment 4, Table 4—Behavior of a Torula Species, S. cerevisiae, and S. ellipsoideus Hansen in Loganberry Juice and Loganberry Juice-Sucrose Solutions: These yeast cells tended to survive several weeks of storage in greater numbers in the solutions containing the higher concentrations of sucrose; however, survival was not consistently higher in 60-per cent sucrose media than in 50-per cent. It is conceivable that any increase in sucrose content beyond a certain percentage might exert a harmful influence on cell viability. For a specific set of conditions the percentage of sucrose which might exert a harmful influence would probably vary

TABLE 2

Behavior of Escherichia coli in Orange Juice and in Orange Juice-Sucrose Solutions Stored at —17.8°C.(0°F.)

Medium	Dextrose agar plate counts (per ml.)							
	Before freezing	After freezing						
		1 wk.	2 wks.	4 wks.	6 wks.	8 wks.	16 wks.	26 wks.
Juice	8,400,000	3	(?) + [2]	(?) +	(?) +	.....	.....	.....
J + suc. to 20% [1]	5,800,000	220,000	1,900	1,500	373	13	.....	.....
J + suc. to 30%	5,600,000	1,157,000	1,241,000	893,000	230,000	130,000	19,300	(?) +
J + suc. to 40%	3,800,000	2,673,000	1,582,000	1,677,000	470,000	590,000	237,000	7,960
J + suc. to 50%	4,000,000	3,340,000	1,492,000	1,683,000	915,000	505,000	563,000	9,320

[1] J + suc. to 20% means that sucrose has been added to juice in a quantity sufficient to give a total soluble-solids content of 20 per cent.

[2] (?) Doubtful positives.

TABLE 3

Behavior of a Torula Species in Orange Juice and in Orange Juice-Sucrose Solutions Stored at —17.8°C.(0°F.)

Medium	Dextrose agar plate counts (per ml.)							
	Before freezing	After freezing						
		1 wk.	8 wks.	16 wks.	24 wks.	32 wks.	40 wks.	52 wks.	72 wks.
Juice	805,000	753,000	590,000	392,000	198,500	18,350	5,984	1,035	202
J + suc. to 20% [1]	720,000	737,000	640,000	520,000	371,500	175,000	106,500	52,000	1,489
J + suc. to 30%	740,000	673,000	693,000	782,000	616,500	380,000	363,500	228,000	8,667
J + suc. to 40%	693,000	667,000	690,000	679,000	580,000	518,500	450,000	342,500	50,000
J + suc. to 50%	660,000	637,000	745,000	715,000	661,500	623,500	587,000	562,500	356,000

[1] J + suc. to 20% means that sucrose has been added to juice in a quantity sufficient to give a total soluble-solids content of 20 per cent.

with the nature of the suspended microorganism, and might be influenced by the number of cells in suspension. The total numbers of cells which remained viable in each medium after the entire storage period are listed (Table 4). These values represent, for the Torula species (given in order of increasing sucrose concentrations of the solutions), less than .1, .48, 3.27, 6.00, 8.01, and 3.14 per cent, respectively, of the original numbers of viable cells contained in the media at the time of freezing; for *S. cerevisiae*,

TABLE 4

Behavior of a Torula Species, Saccharomyces cerevisiae, and Saccharomyces ellipsoideus in Loganberry Juice and in Loganberry Juice-Sucrose Solutions Stored at —17.8°C.(0°F.)

Medium	Wort agar plate counts (per ml.)							
	Before freezing	After freezing						
		1 wk.	2 wks.	4 wks.	8 wks.	12 wks.	16 wks.	20 wks.
Torula species								
Juice	7,000	550	355	227	69	18	7	1
J + suc. to 20%[1]	6,230	800	590	425	225	134	51	30
J + suc. to 30%	4,650	1,065	755	645	355	332	242	152
J + suc. to 40%	5,080	1,400	1,000	678	510	410	280	305
J + suc. to 50%	5,530	1,925	1,450	710	700	425	425	443
J + suc. to 60%	7,000	1,200	1,375	770	450	320	215	220
S. cerevisiae								
Juice	9,800	3,075	1,700	700	77	9	1	0
J + suc. to 20%	9,300	3,775	2,700	1,225	515	180	50	19
J + suc. to 30%	9,100	3,425	2,725	1,750	770	440	184	99
J + suc. to 40%	9,600	4,250	2,525	1,950	1,080	665	330	182
J + suc. to 50%	9,300	5,150	3,875	2,325	1,160	735	448	303
J + suc. to 60%	9,100	5,600	4,425	2,975	1,065	900	350	275
S. ellipsoideus								
Juice	10,800	650	335	54	7	0	0	.....
J + suc. to 20%	10,600	2,075	1,000	373	84	35	10	.....
J + suc. to 30%	6,900	2,150	1,325	628	360	146	93	.....
J + suc. to 40%	7,600	2,100	1,470	805	445	300	176	.....
J + suc. to 50%	10,100	3,900	2,575	1,395	775	425	290	.....
J + suc. to 60%	11,000	4,575	3,525	2,290	1,265	685	443	.....

[1]J + suc. to 20% means that sucrose has been added to juice in a quantity sufficient to give a total soluble-solids content of 20 per cent.

0, .20, 1.09, 1.90, 3.26, and 3.02 per cent, respectively; and for *S. ellipsoideus*, 0, .09, 1.35, 2.32, 2.87, and 4.03 per cent, respectively.

A second experiment was set up using the Torula species as the test organism. This time the original suspensions at the time of freezing contained 62,000 to 108,000 viable cells per milliliter. Tabulated data are not given but the numbers of viable cells in the different media at the end of 52 weeks of storage were 21 (less than .10 per cent); 4,567 (4.87 per cent); 17,200 (27.74 per cent); 36,500 (42.29 per cent); 82,000 (90.11 per cent); and 112,000 (103.70 per cent). Ninety-nine per cent of the Torula cells were destroyed in the unsweetened juice by the 16th week. The consistently high plate counts given by samples of the stored 60-per cent juice-sucrose suspension reveal that the count made prior to freezing

was low and that destruction was not very great in this medium during the 52-week storage period.

A second experiment was also set up with 23,000 to 61,700 cells per milliliter of *S. ellipsoideus* suspended in the different media. The numbers of viable cells at the end of 32 weeks of storage were 0; 0; 26 (.07 per cent); 219 (.95 per cent); 145 (.32 per cent); and 334 (.65 per cent). Ninety-nine per cent of the cells were destroyed in the unsweetened juice by the second week; in the 20-per cent juice-sucrose solution, by the eighth week; in the 30 per cent, by the 16th week; and in the other juice-sucrose solutions, between the 24th and 32nd weeks.

TABLE 5

Behavior of a Torula Species, Saccharomyces cerevisiae, and Saccharomyces ellipsoideus in Loganberry Juice and in Acidified Loganberry Juice-Sucrose Solutions Stored at —17.8°C.(0°F.)

Medium	Wort agar plate counts (per ml.)							
	Before freezing	After freezing						
		1 wk.	2 wks.	4 wks.	8 wks.	12 wks.	16 wks.	30 wks.
Torula species								
Juice	14,333	1,065	253	129	41	19	5	0
J + suc. to 20%[1]	13,133	2,985	1,640	977	535	427	310	46
J + suc. to 30%	7,667	2,300	1,565	1,084	830	613	450	154
J + suc. to 40%	5,933	2,700	2,060	1,527	1,130	957	700	327
J + suc. to 50%	5,000	2,915	2,340	2,254	1,450	1,103	980	602
J + suc. to 60%	7,767	3,615	3,570	2,364	1,950	1,437	1,075	563
S. cerevisiae								
Juice	12,667	5,567	2,450	690	95	16	3	0
J + suc. to 20%	10,867	7,300	5,200	2,275	925	487	188	10
J + suc. to 30%	10,000	6,767	5,100	3,110	1,695	1,090	695	156
J + suc. to 40%	11,833	8,733	5,650	3,393	2,225	1,393	1,100	313
J + suc. to 50%	11,000	10,600	6,350	2,433	2,110	1,567	1,010	360
J + suc. to 60%	11,267	10,033	6,125	3,600	1,975	1,353	785	277
S. ellipsoideus								
Juice	7,733	133	14	1	(?)0[2]	.....	.....	.....
J + suc. to 20%	6,700	865	385	64	4	(?)1	.....	.....
J + suc. to 30%	4,933	1,100	670	285	83	22	12	.....
J + suc. to 40%	6,167	1,085	625	240	81	41	17	.....
J + suc. to 50%	7,167	2,100	1,350	598	180	78	32	.....
J + suc. to 60%	6,167	2,165	1,590	838	390	111	35	.....

[1]J + suc. to 20% means that sucrose has been added to juice in a quantity sufficient to give a total soluble-solids content of 20 per cent. [2](?) Doubtful.

Experiment 5, Table 5—Behavior of a Torula Species, S. cerevisiae, and S. ellipsoideus Hansen in Loganberry Juice and Acidified Loganberry Juice-Sucrose Solutions: Even though the total acidities of the loganberry juice-sucrose solutions were altered to approximate that of unsweetened juice (Table 1) greater numbers of yeast cells survived in the solutions containing the higher concentrations of sucrose than in unsweetened juice or in 20-per cent to 30-per cent juice-sucrose solutions. The relative values of the 50-per cent and the 60-per cent media for retaining yeast cell viability over long storage periods at —17.8°C. were not determined. Neither was the possible influence of the storage-time factor investigated. The last

plate counts recorded (Table 5) represent, for the Torula species, 0, .35, 2.01, 5.51, 12.04, and 7.25 per cent, respectively, of the viable cell counts in the different media at the time of storage; for *S. cerevisiae*, 0, less than .10, 1.56, 2.65, 3.27, and 2.46, respectively; and for *S. ellipsoideus*, —,—, .24, .28, .45, and .57, respectively.

DISCUSSION

Although the studies described were concerned with fruit juice and fruit juice-sucrose solutions, it is believed that the data obtained are useful for interpreting microbial behavior in dry sucrose and in sucrose-sirup-packed fruits since in these packs also the microbial environment for the most part consists of water, sucrose, and soluble fruit solids in varying combinations. The data are not intended to serve as a basis for any recommendation that sucrose be used in the freezing preservation of fruit juices. On the contrary the data clearly indicate that from a microbiological standpoint sucrose offers no advantage in the freezing preservation of fruit juices except to assist in the maintenance of desirable fresh-fruit characteristics and except when the microbial count can be otherwise controlled. In these studies primary consideration has been given to the yeasts since they are the microorganisms chiefly responsible for spoilage in airtight, frozen-pack containers.

The experimental findings bear out previous observations and expressed suspicions that high concentrations of sucrose tend to protect microorganisms during low-temperature storage. Since the experiments were very time-consuming, it was not possible to set up a sufficient number to give all the desired information. Further research is needed to determine the following: (1) if there is a concentration of sucrose which will give maximum protection to a specific microorganism at —17.8°C.(0°F.) but which if increased or decreased will exert a proportionately detrimental influence; and (2) the exact influence on microbial survival in stored sucrose-containing media of such factors as heaviness of the initial contamination or inoculum, nature of the microorganism, volume of the mass, freezing and storage temperatures, method of freezing, and duration of storage. At present one may only speculate as to the nature of the influence which sucrose might exert on microbial survival at —17.8°C. if a perfectly uniform distribution of soluble solids and suspended particles could be maintained throughout the frozen mass. Perhaps one cannot hope to overcome all factors favoring microbial survival in frozen-pack fruits and juices. Nevertheless the employment of sanitary methods from time of harvesting to consumption of the product and the intelligent application of those processing and handling methods which tend to lower the microbial count and delay the development of the remaining viable forms may markedly reduce potential spoilage possibilities and likewise the accompanying worries.

SUMMARY

Suspensions of *E. coli*, *B. subtilis* spores, and a Torula species were made in unsweetened orange juice and in orange juice-sucrose solutions (containing 20, 30, 40, and 50 per cent soluble solids). Similarly, suspensions of a Torula species, *S. cerevisiae*, and *S. ellipsoideus* Hansen were made in unsweetened loganberry juice, in loganberry juice-sucrose solu-

tions (containing 20, 30, 40, 50, and 60 per cent soluble solids), and in loganberry juice-sucrose solutions to which citric acid had been added so that the total acidity of each medium approximated that of the unsweetened juice. Ten-milliliter samples of each suspension were frozen and stored at —17.8°C.

Greater microbial destruction occurred in the unsweetened juice than in the juice-sucrose media stored at —17.8°C. The total numbers of viable cells surviving several weeks of storage in the juice-sucrose media tended to vary as the concentrations of the sucrose. In some of the experimental series the highest concentrations of sucrose, 50 and 60 per cent, did not always give the greatest protection; however, the limited experimental data did not permit conclusions regarding this observation.

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